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(57) Abstract

The present invention is directed to isolated genomic sequences that are differentially expressed within seed–coat tissues. These DNAs are expressed within the inner integument, thick, or thin, walled parenchyma, endothelium, palisade, hourglass, or stellate parenchyma cells of the seed–coat. Furthermore, this invention relates to promoter regions obtained from genomic sequences that are differentially expressed in seed–coat tissues, and their use for directing seed–coat specific expression of genes of interest within transformed plant cells or plants. Exemplified promoters include those obtained from the differential screening of a seed–coat library and cryptic promoters obtained using T–DNA tagging using a promoterless marker gene.

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SEED-COAT PROMOTERS, GENES AND GENE PRODUCTS

Field of Invention

This invention relates to seed-coat promoters, genes and proteins encoded by these genes. More specifically, it relates to genes and promoters that are developmentally regulated and expressed, or activated, within tissues comprising the seed-coat, and tissues directly associated with the seed-coat, of plants. Furthermore, this invention also relates to proteins encoded by genes expressed within these tissues are their localization within, or onto, the seed-coat.

Background and Prior Art

Bacteria from the genus *Agrobacterium* have the ability to transfer specific segments of DNA (T-DNA) to plant cells, where they stably integrate into the nuclear chromosomes. Analyses of plants harbouring the T-DNA have revealed that this genetic element may be integrated at numerous locations, and can occasionally be found within genes. One strategy which may be exploited to identify integration events within genes is to transform plant cells with specially designed T-DNA vectors which contain a reporter gene, devoid of *cis*-acting transcriptional and translational expression signals (i.e. promoterless), located at the end of the T-DNA. Upon integration, the initiation codon of the promoterless gene (reporter gene) will be juxtaposed to plant sequences. The consequence of T-DNA insertion adjacent to, and downstream of, gene promoter elements may be the activation of reporter gene expression. The resulting hybrid genes, referred to as T-DNA-mediated gene fusions, consist of unknown and thus uncharacterized plant promoters residing at their natural location within the chromosome, and the coding sequence of a marker gene located on the inserted T-DNA (Fobert *et al.*, 1991, Plant Mol. Biol. 17, 837-851).

It has generally been assumed that activation of promoterless or enhancerless marker genes result from T-DNA insertions within or immediately adjacent to genes. The recent isolation of several T-DNA insertional mutants (Koncz *et al.*, 1992, *Plant Mol. Biol.* **20**, 963-976; reviewed in Feldmann, 1991, *Plant J.* **1**, 71-82; Van Lijsebettens *et al.*, 1991, *Plant Sci.* **80**, 27-37; Walden *et al.*, 1991, *Plant J.* **1**: 281-288; Yanofsky *et al.*, 1990, *Nature* **346**, 35-39), shows that this is the case for at least some insertions. However, other possibilities exist. One of these is that integration of the T-DNA activates silent regulatory sequences that are not associated with genes. Lindsey *et al.* (1993, *Transgenic Res.* **2**, 33-47) referred to such sequences as "pseudo-promoters" and suggested that they may be responsible for activating marker genes in some transgenic lines.

Inactive regulatory sequences that are buried in the genome but with the capability of being functional when positioned adjacent to genes have been described in a variety of organisms, where they have been called "cryptic promoters" (Al-Shawi *et al.*, 1991; *Mol. Cell. Biol.* **11**, 4207-4216; Fourel *et al.*, 1992, *Mol. Cell. Biol.* **12**, 5336-5344; Irniger *et al.*, 1992, *Nucleic Acids Res.* **20**, 4733-4739; Takahashi *et al.*, 1991, *Jpn J. Cancer Res.* **82**, 1239-1244). Cryptic promoters can be found in the introns of genes, such as those encoding for yeast actin (Irniger *et al.*, 1992, *Nucleic Acids Res.* **20**, 4733-4739), and a mammalian melanoma-associated antigen (Takahashi *et al.*, 1991, *Jpn J. Cancer Res.* **82**, 1239-1244). It has been suggested that the cryptic promoter of the yeast actin gene may be a relict of a promoter that was at one time active but lost function once the coding region was assimilated into the exon-intron structure of the present-day gene (Irniger *et al.*, 1992, *Nucleic Acids Res.* **20**, 4733-4739). A cryptic promoter has also been found in an untranslated region of the second exon of the woodchuck N-myc proto-oncogene (Fourel *et al.*, 1992, *Mol. Cell. Biol.* **12**, 5336-5344). This cryptic promoter is responsible for activation of a N-myc2, a functional processed gene which arose from retroposition of N-*myc* transcript (Fourel *et al.*, 1992, *Mol.*

Cell. Biol. **12**, 5336-5344). These types of regulatory sequences have not yet been isolated from plants.

Weber et al. (1995, Plant Cell 7:1835-1846) disclose a cDNA sequence of a seed-coat associated invertase. However, all of the cDNA's characterized were found to be expressed in tissues other than the seed-coat, including anthers, cotyledon, stem and root. Furthermore, no promoter was isolated, characterized, or disclosed.

Described herein is the occurrence of seed-coat genes and promoters that have been obtained as a result of differential screening of seed-coat genomic libraries, or generated by tagging with a promoterless GUS (β-glucuronidase) T-DNA vector, or by identification of genes that are highly expressed in the seed-coat or associated tissues. Expression analysis of these DNA's reveal that they are spatially and developmentally regulated in seed coats. Prior to this work, promoters, as well as genes specifically expressed in seed coat tissues had not been isolated or reported. Furthermore, proteins encoded by genes that are expressed within seed-coat, or associated with seed-coat tissues, are also disclosed.

Summary of Invention

This invention relates to seed-coat promoters and genes. More specifically, it relates to genes and promoters that are developmentally regulated and expressed, or activated, within tissues comprising the seed-coat of plants, and tissues directly associated with the seed-coat, of plants. Furthermore, this invention also relates to proteins encoded by genes expressed within these tissues and their localization within, or onto, the seed-coat..

A transgenic tobacco plant, T218, contained a 4.7 kb *Eco*RI fragment containing the 2.2 kb promoterless GUS-*nos* gene and 2.5 kb of 5' flanking tobacco DNA. Deletion of the region approximately between 2.5 and 1.0 kb of the 5' flanking region did not alter GUS expression, as compared to the entire 4.7 kb GUS fusion. A further deletion to 0.5 kb of the 5' flanking site resulted in complete loss of GUS activity. Thus the region between 1.0 and 0.5 of the 5' flanking region of the tobacco DNA contains the elements essential to gene activation. This region is contained within a *Xba*I - *Sna*BI restriction site fragment of the flanking tobacco DNA. Furthermore, other promoters have been identified that are differentially expressed within the seed-coats of plants, and that are capable of driving expression of heterologous genes that are operatively linked thereto.

Thus according to the present invention there is provided an isolated genomic DNA molecule, differentially expressed in seed coat tissues. Furthermore, this genomic DNA molecule is differentially expressed within the outer integument of the seed coat, the inner integument of the seed coat, the thick walled parenchyma of the seed coat, the thin walled parenchyma of the seed coat, the endothelium of the seed coat, the hourglass cells of the seed coat, the palisade of the seed coat, the stellate parenchyma of the seed coat, or the membranous endocarp, or a combination thereof.

This invention is also directed to a seed-coat promoter obtained from the genomic DNA molecule as described above. Also considered within the scope of the present invention is a cryptic seed coat promoter. Furthermore, this invention is directed to a seed coat promoter, as described above, that controls the differential expression of a gene associated therewith, within the outer integument of the seed coat, the inner integument of the seed coat, the thick walled parenchyma of the seed coat, the thin walled parenchyma of the seed coat, the endothelium of the seed coat, the hourglass cells of the seed coat, the

palisade of the seed coat, or the stellate parenchyma the seed coat, membranous endocarp, or a combination thereof

This invention also relates to an isolated genomic DNA characterized by the restriction map selected from the group consisting of Figure 12 (a), Figure 12 (b), Figure 12 (c) and Figure 12 (d).

According to the present invention, there is also provided an isolated seed-coat promoter. Furthermore, this seed coat promoter may be obtained from angiosperms. More specifically, this seed-coat promoter is obtained from the group consisting of tobacco or soybean.

This invention is also directed to a cloning vector comprising a gene encoding a protein and an isolated seed-coat promoter, wherein the gene is under the control of the seed-coat promoter. Furthermore, this invention includes a plant cell which has been transformed with such a vector.

This invention also provides for a transgenic plant containing a seed-coat promoter, operatively linked to a gene encoding a protein.

The present invention is also directed to a seed-coat promoter comprising at least 10 contiguous nucleotides of nucleotides 1-2526 of SEQ ID NO:7, or an analogue of the sequence defined by nucleotides 1-2526 of SEQ ID NO:7, wherein the analogue hybridizes to a nucleic acid defined by nucleotides 1-2526 of SEQ ID NO:7 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

This invention also includes a seed-coat promoter comprising at least 10 contiguous nucleotides of nucleotides 1-2450 of SEQ ID NO:8, or an analogue of the nucleic acid sequence defined by nucleotides 1-2450 of SEQ ID NO:8, wherein the analogue hybridizes to a nucleic acid defined by nucleotides

1-2450 of SEQ ID NO:8 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

The present invention also is directed to a seed-coat promoter comprising at least 10 contiguous nucleotides of nucleotides 1-5514 of SEQ ID NO:9, or an analogue of the nucleotides sequence defined by nucleotides 1-5514 of SEQ ID NO:9, wherein the analogue hybridizes to a nucleic acid defined by nucleotides 1-5514 of SEQ ID NO:9 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

Brief Description of the Drawings

Figure 1 depicts the fluorogenic analyses of GUS expression in the plant T218. Each bar represents the average ± one standard deviation of three samples. Nine different tissues were analyzed: leaf (L), stem (S), root (R), anther (A), petal (P), ovary (O), sepal (Se), seeds 10 days post anthesis (S1) and seeds 20 days post-anthesis (S2). For all measurements of GUS activity, the fraction attributed to intrinsic fluorescence, as determined by analysis of untransformed tissues, is shaded black on the graph. Absence of a black area at the bottom of a histogram indicates that the relative contribution of the background fluorescence is too small to be apparent.

Figure 2 shows the cloning of the GUS fusion in plant T218 (pT218) and construction of transformation vectors. Plant DNA is indicated by the solid line and the promoterless GUS-*nos* gene is indicated by the open box. The transcriptional start site and presumptive TATA box are located by the closed and open arrow heads respectively. DNA probes #1, 2, 3 and RNA probe #4 are shown. The *Eco*RI fragment in pT218 was subcloned in the pBIN19 polylinker to create pT218-1. Fragments truncated at the *Xba*I *Sna*BI and *Xba*I sites were also subcloned to create pT218-2, pT218-3 and pT218-4.

Abbreviations for the endonuclease restriction sites are as follows: *Eco*RI (E), *Hin*dIII (H), *Xba*I (X), *Sna*BI (N), *Sma*I (M), *Sst*I (S).

Figure 3 shows the expression pattern of promoter fusions during seed development. GUS activity in developing seeds (4-20 days postanthesis (dpa)) of (Fig. 3a) plant T218 (●-●) and (Fig. 3b) plants transformed with vectors pT218-1 (O-O), pT218-2 (□-□), pT218-3 (▽-▽) and pT218-4 (Δ-Δ) which are illustrated in Figure 2. The 2 day delay in the peak of GUS activity during seed development, seen with the pT218-2 transformant, likely reflects greenhouse variation conditions.

Figure 4 shows GUS activity in 12 dpa seeds of independent transformants produced with vectors pT218-1 (O), pT218-2 (□), pT218-3 (▽) and pT218-4 (Δ). The solid markers indicate the plants shown in Figure 3 (b) and the arrows indicate the average values for plants transformed with pT218-1 or pT218-2.

Figure 5 shows the mapping of the T218 GUS fusion termini and expression of the region surrounding the insertion site in untransformed plants. Figure 5(a) shows the mapping of the GUS mRNA termini in plant T218. The antisense RNA probe from subclone #4 (Figure 2) was used for hybridization with total RNA of tissues from untransformed plants (10 μg) and from plant T218 (30 μg). Arrowheads indicate the anticipated position of protected fragments if transcripts were initiated at the same sites as the T218 GUS fusion. Figure 5 (b) shows the RNase protection assay using the antisense (relative to the orientation of the GUS coding region) RNA probe from subclone e (Figure 7) against 30 μg total RNA of tissues from untransformed plants. P, untreated RNA probe; -, control assay using the probe and tRNA only; L, leaves from untransformed plants; 8, 10, 12, seeds from untransformed plants at 8, 10, and 12 dpa, respectively; T10, seeds of plant T218 at 10 dpa; +, control hybridization against unlabelled *in vitro*-synthesized sense RNA from subclone

c (panel a) or subclone e (panel b). The two hybridizing bands near the top of the gel are end-labelled DNA fragment of 3313 and 1049 bp, included in all assays to monitor losses during processing. Molecular weight markers are in number of bases.

Figure 6 provides the nucleotide sequence of pT218 (top line) (SEQ ID NO: 1) and pIS-1 (bottom line). Sequence identity is indicated by dashed lines. The T-DNA insertion site is indicated by a vertical line after bp 993. This site on pT218 is immediately followed by a 12 bp filler DNA, which is followed by the T-DNA. The first nine amino acids of the GUS gene and the GUS initiation codon (*) are shown. The major and minor transcriptional start site is indicated by a large and small arrow, respectively. The presumptive TATA box is identified and is in boldface. Additional putative TATA and CAAT boxes are marked with boxes. The location of direct (1-5) and indirect (6-8) repeats are indicated by arrows.

Figure 7 shows the base composition of region surrounding the T218 insertion site cloned from untransformed plants. The site of T-DNA insertion in plant T218 is indicated by the vertical arrow. The position of the 2 genomic clones pIS-1 and pIS-2, and of the various RNA probes (a-e) used in RNase protection assays are indicated beneath the graph.

Figure 8 shows the Southern blot analyses of the insertion site in *Nicotiana* species. DNA from *N. tomentosiformis* (N tom), *N. sylvestris* (N syl), and *N. tabacum* (N tab) were digested with *Hin*dIII (H), *Xba*I (X) and *Eco*RI (E) and hybridized using probe #2 (Figure 2). Lambda *Hin*dIII markers (kb) are indicated.

Figure 9 shows the AT content of 5' non-coding regions of plant genes. A program was written in PASCAL to scan GenBank release 75.0 and to calculate the AT contents of the 5' non-coding (solid bars) and the coding

regions (hatched bars) of all plant genes identified as "Magnoliophyta" (flowering plants). The region -200 to -1 and +1 to +200 were compared. Shorter sequences were also accepted if they were at least 190 bp long. The horizontal axis shows the ratio of the AT content (%). The vertical axis shows the number of the sequences having the specified AT content ratios

Figure 10 shows a Northern analysis of the expression of several of the genes of the present invention within developing seed coats, embryo, pod, flower, root, stem and leaf tissues. Figure 10 (a) shows the expression of SC4; Figure 10 (b) shows the expression of SC20; Figure 10 (c) shows the expression of SC21, Figure 10 (d) shows the expression of *Ep* locus peroxidase within these tissues. Figure 10 (e) shows the expression of HP (hydrophobic protein) in leaf, flower, pod, seed coat, embryo, stem or root tissues. Figures 10 (f) and (g), total RNA was isolated from leaf, flower, pod shells, seed coat, embryo, stem or root tissue. Equal amounts of RNA (10 μg) were vacuum blotted to nylon and probed with *HPS* cDNA. Ribosomal RNA (rRNA), visualized by staining with ethidium bromide, is shown as control. Figure 10 (f), RNA from tissues at early (E) mid (M) or late (L) stages of development were compared for *HP* gene expression. All samples shown are from dull seeded phenotype (cv Harosoy 63). Figure 10 (g), RNA from pod tissues of dull (cv Harosoy 63) and shiny (cv. Williams 82) seeded soybeans were compared for *HP* gene expression.

Figure 11 shows the restriction maps obtained from Figure 11 (a) SC20; Figure 11 (b) SC21; Figure 11 (c) HP (hydrophobic protein) genomic region, and Figure 11 (d) SC4. Included in Figure 11 (c) are restriction enzyme sites for *BamH*I, *Bgl*II, *Hind*III, and *Xba*I; the HP ORF; TATA box consensus signals; and the position of direct repeats of 12 bp or longer.

Figure 12 shows the morphology of the seed coat of *Glycine max*. Figure 12 (a) shows the structures present at six days after anthesis (DAF); Figure 12 (b), at 12 DAF; and Figure 12 (c) at 18 DAF.

Figure 13 shows *in situ* hybridization results obtained with seed coats of *Glycine max* at different developmental stages, and probed as follows: Figure 13 (a) seed coat at 3 days after anthesis (DAF), probed with SC4; Figure 13 (b) seed coat at 9 DAF, probed with SC20; Figure 13 (c) seed coat at 15 DAF, probed with SC21; Figure 13 (d) seed coat at 18 days after anthesis, probed with a soybean peroxidase, corresponding to the Ep locus. Figures 13 (e), (f) and (g) were obtained from cross sections of developing soybean seeds (cultivar Maple Presto, *EpEp*). Hybridization of ^{35}S-probe to complementary mRNA appears as bright white signal in these dark field microscopy images. Figure 13 (e) 6 DAF (DPA, days post anthesis), Figure 13 (f) 9 DAF, and Figure 13 (g) 12 DAF. Scale bars are 100 :m. Emb, embryo; F, funiculus; HG, hourglass cells; PC, pericarp; SC, seed coat.

Figure 14 shows light micrographs of a seed-coat obtained from soybean. Figure 14 (a) shows a plastic embedded section of the seed-coat near the hilum at 21 daf and stained with Toluidine Blue O. Note the association of the membranous endocarp with the seed-coat pallisade. Figure 14 (b) shows a wax-embedded section of a soybean seed-coat as 12 daf probed with ^{35}S-labelled Hydrophobic Protein (HP) antisense RNA, and counter stained with Toluidine Blue O. Note strong specific localization of the probe within the membranous endocarp. Pallisade (p), hourglass cells (h), counterpallisade (c), arial cells (a), stellate parenchyma (s), thin walled parenchyma (n), thickwalled parenchyma (k), pod parenchyma (d), and membranous endocarp ➤. Figures 14 (c) and (d). show localization of *HP* mRNA transcript by *in situ* hybridization. Cross sections of soybean pods containing immature seeds (dull phenotype, HPS (+), cv Maple Presto). Hybridization of ^{35}S labelled *HP* probe to complementary mRNA appears as bright white signal in these dark field

microscopy images. E, embryo; Ep, inner epidermal layer of endocarp; Ex, exocarp; F, funiculus; M, mesocarp; Sc, seed coat; Sm, sclerenchyma layer of endocarp. Bar = 100 μm. Figure 14 (c), Expression at 6 DPA (days post anthesis). Figure 14 (d), Expression 12 DPA.

Figure 15 shows the Soybean hydrophobic protein (HP) cDNA and deduced amino acid sequences. Figure 15 (a), the cDNA and amino acid sequence of HP. The pre-protein signal sequence is underlined. Figure 15 (b) shows the deduced amino acid sequence of HP pre-protein. Alternate N-terminal residues are boxed, as determined by peptide microsequence analysis. Figure 15 (c) shows a Kyle-Doolittle hydrophilicity plot of HP (Lasergene). In this plot, positive values indicate greater hydrophilic character. Also represented are the three domains of the HP pre-protein and the length of the mature peptide. Figure 15 (d) shows a schematic comparison of HP domain structure to three other plant proteins. Bold numbers indicate the length in amino acid residues for the domain segments. The pattern of spacing between the eight cysteine residues within the hydrophobic domains is also shown below each protein. Sequences for tobacco N16 polypeptide (D86629), maize proline rich hydrophobic protein (PRHP) (X60432), and *Arabidopsis* lipid transfer protein 1 (LTP1) (M80567) were retrieved from GenBank.

Figure 16 shows scanning electron micrographs of representative 'Dull' and 'Shiny' seeded soybean cultivars. Scale bars are included in the figures. The lowest magnification (x18), Figure 16 (a) is a view of the entire seed. The large oval shaped scar on the seed surface is the hilum, corresponding to the point of detachment of the mature seed from the funiculus. Figure 16 (b), x100, and Figure 16 (c) x500, are focused outside of hilum region.

Figure 17 shows a silver stained SDS-PAGE analysis of protein extracts from seed tissues and surface. Lanes marked 'M' indicate protein standards, and their corresponding mass in kilodaltons is also provided. Figure 17 (a),

Soluble protein extracts from the embryo, seed coat, and seed surface of a dull phenotype (cv Harosoy 63). Each sample at approximately 1 μg of total protein. Figure 17 (b), Seed surface protein extracts of a dull phenotype (cv Harosoy 63) with different concentrations of dithiothreitol (DTT) present in the sample loading buffer, as indicated at the top of each lane. Figure 17 (c), Seed surface protein extracts of dull (D), shiny (S), and bloom (B).

Figure 18 shows restriction fragment length polymorphisms between dull and shiny phenotypes. Genomic DNA from dull (cv Harosoy 63) and shiny (cv Williams 82) soybeans with abundant (+) or trace (-) amounts of HPS on the seed surface, was digested with restriction enzymes, electrophoretically separated, blotted, and hybridized to HP cDNA probe. The size of hybridizing fragments was estimated by comparison with standards and is shown on the left.

Figure 19 shows the nucleotide sequence and deduced amino acid sequence of SC4 cDNA, and the sequence comparisons between SC4 protein and BURP proteins. Figure 19 (a), 5' and 3' untranslated sequences are in lowercase lettering. The stop codon is shown with an asterisk and two polyadenylation signals are underlined. Two copies of a ten amino acid repeat is also underlined. Concensus sequnences for N-glycosylation (NNT; NSSN; and NGTV) are also underlined. Figure 19 (b), amino acid alignment of the carboxyl terminus of the SC4 protein with the BURP domain (A) and the amino terminus of the SC4 protein with the conserved segments of the second domain (B) of several BURP domain proteins. Pg1β is not included in panel B as the second domain of this protein does not contain a conserved segment. Gaps were introduced to optimize the alignment. Conserved amino acids are shown in bold face. Amino acids of each protease are numbered from the precursor sequence. Figure 19 (c) shows the structual similarity between SC4 protein and the BURP domain proteins.

Figure 20 shows Northern blot analysis of SC4 and SC20 mRNA accumulation in seed coat embryo and pod organs of soybean. 10 μg total RNA from seed coat, embryo and pod organs between 6-24 days past anthesis were hybridized with radiolabled probes. For day 6, total RNA was prepared from whole seeds. Each blot was hybridized with a SC4 cDNA probe, Figure 20 (a), a SC20 cDNA probe Figure 20 (b), and an 18S rRNA probe Figure (c).

Figure 21, shows the localization of SC4 mRNA in Seed coat organs of soybean by *in situ* hybridization. Transections of seed coats at 3 days past anthesis (dpa) and 6 dpa. Hybridization to Antisense, Figure 21 (a), and sense, Figure 21 (b) SC4 labelled RNA probes. Abbreviations, II - inner integument, OI outer integument, P pod. Bar equals 100μm.

Figure 22, shows Southern blot analysis of SC4. Figure 22 (a) shows Southern analysis of the gene family composition of *sc*4 in soybean. Figure 22 (b) shows Southern analysis of *sc*4 in diverse plant species. Hybridized filter was washed under conditions of low stringency, twice at 52°C for 15 min in 2x SSC, 0.1%SSC, 0.1% SDS and once at 52°C for 30 min in 0.1x SSC, 0.1% SDS.

Figure 23 reveals the characterization of *sc*20 and the SC20 protein. Figure 23 (a) is a restriction map of *sc*20. Figure 23 (b) shows the nucleotide sequence and deduced amino acid sequence of sc20 cDNA. The stop codon is shown with an asterisk and the polyadenylation signal is underlined. The concensus sequences for N-glycosylation are also underlined. Figure 23 (c) shows the hydrophobic plot of SC20 protein, where hydrophobic regions possess a positive sign, and hydrophilic regions possess a negative sign. In Figure 23 (d), alignment of SC20 protein with other subtilases is shown. D, H and S regions represent amino acid sequences around the catalytic aspartate, histidine and serine residues of the subtilases. The catalytic residues are labelled with an asterisk. N region represents amino acid sequence around the

conserved asparagine residue, of subtilases. # indicates the conserved asparagine. AF70, cucumisin, P69B, Ag12, subtilisin BPN', kex2, furin are from *Picea abies*, *Cucumis melo* L., *Lycopersicon esculentum*, *Alnus glutinosa*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Homo sapiens* respectively. Conserved amino acids are shown in boldface. Amino acids of each protease are numbered from the precursor sequence.

Figure 24 shows localization of SC20 mRNA in seed coats of soybean by in situ hybridization. Transection of seed coats at 12 days past anthesis hybridized to Antisense, Fivure 24 (a), and Sense, figure 24 (b), SC20 radiolabelled RNA probes. Abbreviations: H Hilumi, II inner integument, OI outer integument, * thick walled parenchyma, ** thin walled parenchyma. Bar equals 100μm.

Figure 25 shows Southern blot analysis of *sc*20. Figure 25 (a) and (b), Southern analysis of the gene family composition of *sc*20 in soybean under conditions of medium stringency (twice at 52°C for 15 min in 2x SSC, 0.1%SDS, and once at 52°C for 30 min in 0.1x SSC, 0.1% SDS), Figure 25 (a), and high stringency (once at 62°C for 30 min in 0.1x SSC, 0.1% SDS) Figure 25 (b). Figure 25 (c) shows Southern analysis of *sc*4 in diverse plant species. genomic DNA was digested with EcoRI. Hybridization used a radiolabelled SC20 cDNA probe. The filter was washed under conditions of medium stringency, twice at 52°C for 15 min in 2x SSC, 0.1% SDS and once at 52°C for 30 min in 0.1x SSC, 0.1% SDS.

Detailed Description of the Preferred Embodiments

T-DNA tagging with a promoterless β-glucuronidase (GUS) gene generated a transgenic *Nicotiana tabacum* plant that expressed GUS activity only in developing seed coats. Cloning and deletion analysis of the GUS fusion

revealed that the promoter responsible for seed coat specificity was located in the plant DNA proximal to the GUS gene. Deletion analyses localized the cryptic promoter to an approximately 0.5 kb region between a *Xba*I and a *Sna*BI restriction endonuclease site of the 5' flanking tobacco DNA. This region spans from nucleotide 1 to nucleotide 467 of SEQ ID NO: 1.

Other work, based on the differential screening of seed coat libraries has led to the identification of several other genes that are differentially expressed within, or tissues that are directly associated with, the seed coat of plants. These genes include SC4 (SEQ ID NO's: 3 and 9, cDNA and genomic sequences, respectively), SC20 (SEQ ID NO's: 4 and 8, cDNA and genomic sequences respectively), SC21 (SEQ ID NO: 5, cDNA sequence), and their associated promoters (see SEQ ID NO 9 and 8 for promoters of SC 4 and SC20, respectively; also Figure 12). Furthermore, the isolation of genes encoding highly expressed seed coat proteins led to the identification of a seed-coat specific peroxidase from the *Ep* locus and associated promoter (Ep genomic sequence, SEQ ID NO:2), as well as a gene encoding a seed-coat localized hydrophobic protein (HP, cDNA sequence SEQ ID NO:6) and associated promoter (within genomic sequence, SEQ ID NO:7, also see Figure 11 (c)). Thus, the present invention includes promoters, genes and proteins isolated from several plant species, that are preferentially expressed, or specific to seed-coat tissues, as well as promoters, genes and associated proteins obtained from tissues associated with the seed-coat.

The term cryptic promoter means a promoter that is not associated with a gene and thus does not control expression in its native location. These inactive regulatory sequences are buried in the genome but are capable of being functional when positioned adjacent to a gene.

The DNA sequence of an aspect of the present invention includes the DNA sequence of SEQ ID NO: 1, the promoter region within SEQ ID NO: 1

(for example from nucleotide 1 to 476), and analogues thereof. Similarly, another aspect of this invention includes a DNA sequence of SEQ ID NO:2, the promoter region of this sequence (nucleotides 1-1532), and analogues thereof. Another aspect of this invention includes a DNA sequence of SEQ ID NO:7, the promoter region (nucleotides 1-2526), and analogues thereof, a DNA sequence of SEQ ID NO 8, the promoter region (nucleotides 1-2450) and analogues thereof, and a DNA sequence of SEQ ID NO:9, the promoter region (nucleotides 1-5514) and analogues thereof.

Analogues include those DNA sequences which hybridize under stringent hybridization conditions (see Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to the DNA sequence of SEQ ID NO: 1, 2, 7, 8 or 9 provided that said sequences maintain the seed coat, or seed-coat associated promoter activity. An example of one such stringent hybridization conditions may be hybridization at 4XSSC at 65°C, followed by washing in 0.1XSSC at 65°C for an hour, or at 62°C for 30 min in 0.1x SSC, 0.1% SDS. Alternatively an exemplary stringent hybridization condition could be in 50% formamide, 4XSSC at 42°C. With the use of Digoxigenin labelled probes, stringent hybridization may include 65 °C in 0.25 M Na_2HPO_4 (pH 7.2), 20% SDS, 1 mM EDTA and 0.5% blocking reagent (Boehringer Mannheim) followed by washing at 22 °C in 20 mM Na_2HPO_4 (pH 7.2), 1% SDS and 1 mM EDTA and washes in the same solution at 68 °C. Analogues also include those DNA sequences which hybridize to the sequence of SEQ ID NO: 1, 2, 7, 8 or 9 under relaxed hybridization conditions provided that said sequences maintain the seed-coat promoter activity. Examples of such non-hybridization conditions includes hybridization at 4XSSC at 50°C or with 30-40% formamide at 42°C. Alternate conditions of medium stringency include washing the filter twice at 52°C for 15 min in 2x SSC, 0.1% SDS and once at 52°C for 30 min in 0.1x SSC, 0.1% SDS.

Furthermore, another aspect of this invention is directed to the identification and characterization of seed-coat promoters (see Figure 11) and their corresponding genes of cDNA's (SEQ ID NO's: 3-6), as characterized by Southern or *in situ* hybridization analysis of the expression patterns of genes expressed under the control of seed-coat promoters within developing seed coats (Figures 13 and 14). Furthermore, restriction maps of the promoter and downstream regions of several seed-coat genomic clones is presented (Figure 11).

Proteins of interest may be expressed in seed coat tissues by placing a gene capable of expressing the protein of interest under the control of the DNA promoters of this invention. Genes of interest include but are not restricted to herbicide resistant genes, genes encoding viral coat proteins, or genes encoding proteins conferring biological control of pest or pathogens such as an insecticidal protein for example *B. thuringiensis* toxin. Other genes include those capable of modifying the production of proteins that alter the taste of the seed and/or that affect the nutritive value of the seed.

By "seed-coat" it is meant tissues typically found within, and associated with, the seed-coat of developing or mature angiosperm seeds. With out wishing to limit the types of tissues found within a seed-coat, this region of the seed typically comprises a range of cell types including, and bounded by, an inner endothelium, and an outer epidermis or palisade cell layer. Within these inner and out cell layers, there may be found parenchyma-like cells, for example thin, or thick walled parenchyma, or stellate parenchyma, vascular tissue, hypodermis, hour-glass cells (osteosclereids), and one or more integuments, including the inner and outer integuments. However, it is to be understood that other cell types found within the region between the inner endothelium and outer epidermis may also be considered to comprise the seed-coat, for example, but not limited to the hilum, and funiculus comprising arial cells. Furthermore, it is to be understood that "seed-coat" also refers to tissues

associated with, or adhering to the seed coat, for example the membranous endocarp (of the inner ovary wall), as this cell type adheres to the seed-coat and remains in association with the seed coat (see for example Figure 15 (a), (b), and Figure 16). Therefore, as used herein, tissues that are associated with, or that adhere to, the seed-coat are referred to as "seed-coat associated tissues", or "tissues associated with the seed-coat" It is contemplated that other cell types may also associate with seed-coat tissues in addition to those disclosed above and that the tissues identified above should not be considered limiting in any manner.

By "seed-coat gene" it is meant a gene that is differentially expressed within the seed-coat as detected under stringent conditions (as defined above). Examples of such a gene include, but are not limited to SC4, SC 20, SC 21, or *Ep* locus peroxidase. However, the product of the gene may be exported from the cell to an exterior location of a seed-coat cell, including the surface of the seed-coat itself. An example, which is not to be considered limiting in any manner, of a gene product that is synthesized in within a seed-coat associated cell, and that is localized onto the surface of the seed coat, is the hydrophobic protein (HP; see Figures 14 (a) (b) and Figure 16).

A "seed-coat promoter" is a promoter that is differentially active within cells of the seed-coat. When operably linked with a gene under its control, a seed-coat promoter confers expression to a gene within the seed-coat, which can be detected under stringent conditions (as defined above). Seed coat associated promoter refers to a promoter that is active in tissue associated with the seed coat as defined above.

By "differentially expressed" it is meant the expression of a gene under the control of a promoter, as detected by standard means, within a specified tissue or organ. Such standard means for detecting expression include, but are not limited to, Northerns, or *in situ* hybridizations and the like performed under

stringent conditions, or reporter gene expression. For example, a gene that is differentially expressed in seed-coat tissues is detectable within seed-coat tissues, and displays little or no expression in other tissues such as root, stem.

By "preferentially expressed" it is meant the expression of a gene under the control of a promoter, as detected by standard means, wherein the majority of expression is detected within a specified tissue or organ. Such standard means for detecting expression include, but are not limited to, Northerns, or *in situ* hybridizations and the like performed under stringent conditions, or the expression of reporter genes. For example, a gene that is preferentially expressed in seed-coat tissues is detectable within seed-coat tissues, but may exhibit some expression within other tissues such as root, stem.

By "seed-coat localized" or "localized onto the seed-coat" it is meant a gene product that, as a result of some property of the amino acid sequence of the gene product, is targeted within, or onto seed-coat tissues, respectively. Properties of an amino acid sequence that may direct the targeting of a protein within or onto seed-coat tissues include, but are not limited to, signal sequences that direct intracellular, and extracellular localization of a protein, and also hydrophobic regions within a protein, that results in localization of the protein onto the seed coat. An example, which is not to be considered limiting, of a protein that is localized onto the seed-coat, is the hydrophobic protein (HP). HP is localized on the outside of the seed-coat following its synthesis within the membranous endocarp, and appears to be involved with the adherence of the endocarp to the seed-coat (see Figure 16).

Development of the Soybean coat

The seed coat of *Glycine max* undergoes dramatic changes in the first two and a half weeks after anthesis (flowering). At six days after anthesis (DAF; see Figure 12 (a)), the seed coat has a distinct epidermis (10), consisting

of thin-walled cuboidal cells; an outer integument (20), consisting of up to a dozen layers of thin-walled parenchyma, and containing vascular tissue (recurrent vascular bundle) in the subhilar region; an inner integument (30), consisting of up to 6 layers of deeply-staining thick-walled parenchyma; and an endothelium (40), consisting of thin-walled cuboidal cells.

At 12 days after anthesis (Figure 12 (b)), there is a distinct hypodermis (15) of thin-walled cuboidal cells directly beneath the epidermis (10); the outer integument (20) has differentiated into an upper layer of thin-walled parenchyma (25), and a lower layer of thick-walled parenchyma (27); the inner integument (30), while still having very thick, deeply staining cell walls, has become stretched, and is compressed to about 3 cells thick; the endothelium (40) is also retained. Also evident in Figure 12 (b) is the endosperm (50), and the developing cotyledons (60).

By 18 days after anthesis (Figure 12 (c)), the epidermal cells have divided and elongated to form thick-walled macrosclereids, forming a palisade layer (13). The hypodermis has differentiated into osteosclereids: thick walled cells with a characteristic I-shape (hourglass cells; 17). A prominent vascular region (70) has developed in the thin-walled parenchyma (25) of the outer integument which stops before reaching the region of the seed opposite the hilum; the thick-walled parenchyma (27) is retained. The inner integument (30) has become completely stretched and crushed, leaving a single, deeply staining wall layer directly above the endothelium (40). The hilum region contains a well-developed counter-palisade (80), and a tracheid bar (90). The seed coat remains attached to the funiculus (100). The sub-hilar region contains well-developed vascular tissue (recurrent vascular bundles; 70) and stellate parenchyma (110).

At maturity, the seed coat consists of the palisade layer (13), hourglass cells (17), a partially crushed layer of parenchyma (what remains of the outer

integument), and an endothelium (40). The remnant of the inner integument (30) is often not distinguishable. The tissues of the hilum although compressed, are retained.

The stages of seed-coat development are also identified in Tables 1 and 2.

Development of the Soybean Seed Coat - Table I

	Epidermis	Hypodermis	Outer Integument	Inner Integument	Endothelium	Status of Embryo
3 daf	simple cuboidal cells	-	simple parenchyma; no vascularization	2-4 layers	simple cuboidal cells	undifferentiated proembryo
6daf	not elongated	-	simple parenchyma; recurrent vascular bundles in subhilar region	5-6 layers; thick walled	simple cuboidal cells	endosperm starting to develop
9daf	starting to divide and elongate	cells starting to differentiate from parenchyma	upper, thinwalled parenchyma with developing vascular region; lower, thickwalled parenchyma; upper and lower parenchyma show some characteristics of aerenchyma	2-3 cell layers, stretched, starting to compress	simple cuboidal cells	cotyledons starting to develop
12 daf	division and elongation	hourglass cells developing	upper, thinwalled parenchyma with extending vascular region; lower, thickwalled parenchyma, more aerenchyma- like characteristics	1-2 cell layers; more compression	cells starting to stretch	cotyledons shifting
15 daf	cell walls thickening	hourglass cells	upper, thinwalled parenchyma with extending vascular region; lower, thickwalled parenchyma, more aerenchyma- like characteristics	layers of deeply staining wall visible	cells stretching	cotyledons expanding
18 daf	palisade (macrosclereids)	hourglass cells (I-shaped osteosclereids)	upper, thinwalled parenchyma with extending vascular region; lower, thickwalled parenchyma, more aerenchyma- like characteristics	compressed; one thick, deeply stained line	cells stretching	cotyledons expanding endosperm compressing; protein accumulation starting
21 daf	palisade (macrosclereids)	hourglass cells	upper, thinwalled parenchyma with vascular region completed; lower, thickwalled parenchyma, more aerenchyma-like characteristics	line is thinning	small, oblong, thick-walled cells	endosperm disappearing, protein and lipid accumulation in cotyledons
24 daf	palisade (macrosclereids)	hourglass cells	upper, thinwalled parenchyma with vascular region; lower, thickwalled parenchyma, more aerenchyma-like characteristics	thinning	small, oblong, thick-walled cells	endosperm no longer distinct, protein and lipid accumulation continue
30 daf	palisade (macrosclereids)	hourglass cells	upper and lower parenchyma starting to compress	thin, deeply stained line visible	small, oblong, thick-walled cells	protein and lipid accumulation slowing
45 daf	palisade (macrosclereids)	hourglass cells	parenchyma partially collapsed, upper and lower parenchyma not distinct, vascular tissue not distinguishable	not distinguishable	small, oblong, thick-walled cells	mature cotyledons

Table 2

Position and Levels of Starch, Protein and Lipid in Relation to Seedcoat Development in Soybean

	Developmental Information	Status of Embryo	Starch Accumulation
1 daf	undifferentiated integument and endothelium distinguishable		throughout seedcoat (also in pod, funiculus & senescing floral parts)
3 daf	inner and outer integument, vascular bundles visible		throughout seedcoat (> in outer integument) except epidermis & recurrent vascular bundles (also in funiculus & pod)
6 daf	inner and outer integument, vascular bundles at hilum	embryo starting to expand	outer integument & stellate parenchyma (pod, trichomes & funiculus)
9 daf	inner integument stretched, vascular bundles expanding away from hilum; outer integument differentiating into an upper and lower region	cotyledons are small and beginning to shift	gradient in epidermis; very few granules in bottom half of seedcoat, stellate parenchyma, outer integument
12 daf	inner integument is crushed, epidermis starting to differentiate into palisade and hypodermis starting to differentiate into hourglass cells	cotyledons have shifted, fill embryonic space	palisade (at hilum), stellate parenchyma, differentiating hourglass cells, outer (outer and inner) and inner integument (funiculus) (starting in cotyledons)
15 daf	epidermis differentiated into palisade and hypodermis differentiated into hourglass cells, upper parenchyma differentiated into upper and lower region		palisade, hourglass cells, stellate parenchyma, outer integument (outer region) (cotyledon epidermis)
18 daf	palisade and hourglass cells fully developed, vascular region is very prominent, seed coat is fully expanded	cotyledons have fully expanded	palisade, hourglass, sparse in stellate, sparse in outer integument (outer and inner) (cotyledons)
21 daf	same as in 18		palisade, sparse in stellate and lower potion of upper parenchyma & vascular parenchyma (*also in layer outside of palisade) (cotyledons)
24 daf	same as in 18		palisade, sparse in lower region of upper parenchyma, starting in endothelium (*also in layer outside of palisade) (cotyledons)
30 daf	outer parenchyma region has disappeared, stellate parenchyma present, vascular regions still present, endothelium prominent		endothelium (also endothelium around hypocotyl)
45 daf	outer parenchyma disappeared, stellate parenchyma present, inner integument collapsed, endothelium very prominent		endothelium (also endothelium around hypocotyl)

Table 2 (cont'd)
Position and Levels of Starch, Protein and Lipid in Relation to Seedcoat Development in Soybean

	Protein Accumulation	Lipid accumulation
1 daf	no distinct protein bodies	
3 daf	no distinct protein bodies	few small lipid droplets in funiculus, nothing in seedcoat
6 daf	no distinct protein bodies	few small droplets in outer integument
9 daf	no distinct protein bodies	small droplets in funiculus and counter palisade, sparse in outer integument
12 daf	no distinct protein bodies in seedcoat but starting in cotyledons	
15 daf	protein bodies in cotyledons getting larger	
18 daf		
21 daf		
24 daf		
30 daf	many large and small protein bodies filling cotyledons	
45 daf		

Early development of the tobacco seed coat

At 6 days after anthesis, the tobacco seed coat consists of an epidermis of very large, thin walled cells; a layer of parenchyma cells up to 6 cells thick; and an endothelium of thin-walled, cuboidal cells. By 10 days after anthesis, the inner walls of the epidermis have thickened significantly, with 2-3 layers discernible; the thin-walled parenchyma has become reduced to 3-4 cells thick due to stretching of the layer as the seed expands; and the endothelial cells have become thinner and elongated. At 22 days after anthesis, the epidermal cells have stretched and elongated to accommodate the expanding seed, and the parenchyma and endothelium have elongated and fused into a crushed layer with few individual cells distinguishable.

Seed-coat cryptic promoter

There are several lines of evidence that suggest that the seed-coat expression of GUS activity in the plant T218 is regulated by a cryptic promoter. The region surrounding the promoter and transcriptional start site for the GUS gene are not transcribed in untransformed plants. Transcription was only observed in plant T218 when T-DNA was inserted in *cis*. DNA sequence analysis did not uncover a long open reading frame within the 3.3 kb region cloned. Moreover, the region is very AT rich and predicted to be noncoding (data not shown) by the Fickett algorithm (Fickett, 1982, *Nucleic Acids Res.* **10**, 5303-5318) as implemented in DNASIS 7.0 (Hitachi). Southern blots revealed that the insertion site is within the *N. tomentosiformis* genome and is not conserved among related species as would be expected for a region with an important gene.

As this is the first report of a cryptic promoter specific to seed-coat tissues in plants, it is impossible to estimate the degree to which cryptic

promoters may contribute to the high frequencies of promoterless marker gene activation in plants. It is interesting to note that transcriptional GUS fusions in *Arabidopsis* occur at much greater frequencies (54%) than translational fusions (1.6%, Kertbundit *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88**, 5212-5216). The possibility that cryptic promoters may account for some fusions was recognized by Lindsey *et al.* (1993, *Transgenic Res.* **2**, 33-47).

The results disclosed herewith confirms others (Gheysen *et al.*, 1987, *Proc. Natl. Acad. Sci. USA* **84**, 6169-6173 and 1991, *Genes Dev.* **5**, 287-297) that T-DNA may insert into A-T rich regions as do plant transposable elements (Capel *et al.*, 1993, *Nucleic Acids Res.* **21**, 2369-2373). We illustrate that promoters of plant genes are also A-T rich raising speculation that gene insertions into these regions could facilitate the rapid acquisition of new regulatory elements during gene evolution.

The insertion of functional genes into the nuclear genome and acquisition of new regulatory sequences has already played a major role in the diversification of certain genes and the endosymbiosis of organelles. In plants, most organellar proteins are nuclear encoded due to the ongoing transfer of their genes into the nucleus (Palmer, 1991, In Bogorad L and Vasil IK (eds) The Molecular Biology of Plastids, Academic Press, San Diego, pp 5-53). Recently, it has been shown that the *cox* 2 gene of cowpea (Nugent and Palmer, 1991, *Cell* **66**, 473-481) and soybean (Covello and Gray, 1992, *EMBO J.* **11**, 3815-3820) were transferred from mitochondria to nucleus without promoters by RNA intermediates. The results disclosed herewith, with T-DNA-mediated gene fusions reveal the facility with which promoters can be acquired by incoming genes. The presence of cryptic promoters and diverse regulatory elements in the intergenic regions may insure that genes rapidly achieve the features needed to meet the demands of complex multicellular organisms.

Other seed-coat, and seed-coat-associated promoters

Transcripts encoding seed coat specific genes were isolated from seed-coat cDNA libraries. These cDNA transcripts were then used to identify the corresponding structural genes and associated promoters from genomic DNA libraries. These promoters, genes and gene products have been isolated and characterized Examples of such genes include, but are not limited to, SC4, SC20, SC21, a peroxidase cloned from the *Ep* locus, and HP (hydrophobic protein). It is to be understood that this seed-coat library comprises tissues typically found within the seed-coat and tissues adhering to the seed-coat such as the membranous endocarp and cells found in the funicular region such as arial cells (see above for full definition of seed-coat).

Ep locus Peroxidase

The amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype. Homozygous recessive *epep* plants are ~100-fold lower in seed coat peroxidase activity which results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the subepidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in the hourglass cells (osteosclereids; which form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces).

A seed-coat peroxidase gene, corresponding to the *Ep* locus, was obtained from a soybean seed-coat library. The genomic DNA sequence comprises four exons spanning bp 1533-1752 (exon I), 2383 -2574 (exon 2), 3605-3769 (exon 3) and 4033-4516 (exon 4) and three introns comprising 1752-2382 (intron 1), 2575-3604 (intron 2) and 3770-4516 (intron 3), of SEQ ID NO:2. Features of the upstream regulatory region of the genomic DNA include a TATA box

centred on bp 1487; a cap signal 32 bp down stream centred on bp 1520. Also noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4663 and a polyadenylation site at bp 4700. The promoter region of the genomic sequence comprises nucleotides 1-1532 of SEQ ID NO:2 (see co-pending US patent application serial No. 08/723,414 and 08/939,905, both of which are incorporated by reference).

Expression of *Ep* is first detected at 6 DPA in the thin-walled parenchyma of the outer integument, adjacent to the thick-walled parenchyma, and flanking the hilum region. By 9 DPA a thin band of expression extends around the entire seed coat, at the junction of the thin-and thick-walled parenchyma. Expression shifts to the hourglass cells as they begin to develop, at 12 DPA (see Figures 13 (e), (f) and (g)).

Expression of a gene under the control of the *Ep* (peroxidase) promoter (nucleotides 1-1532 of SEQ ID NO:2, also see co-pending US patent application serial No. 08/723,414 and 08/939,905, both of which are incorporated by reference) is observed within the seed-coat from 6 to 18 days after anthesis is shown in Figures 13 (d) to (g).

Hydrophobic Protein (HP)

Soybean HP is an 8.3 kD protein consisting of 80 amino acids rich in hydrophobic residues and entirely lacking methionine, phenylalanine, tryptophan, lysine and histidine residues (see Figure 15). The amino acid sequence shows no significant homology to any known proteins (Odani *et al.*, 1987, *Eur J Biochem* 162, 485-491).

To determine the composition of proteins deposited on the soybean seed surface, seeds were washed with a detergent-buffer solution and the extracted peptides were separated by SDS-PAGE. Protein extracts from the seed coat and

embryo were also prepared for comparison. These results are shown in Figure 17 (a). The embryo and seed coat extracts contained many proteins covering a wide molecular mass range. In contrast, extracts from the seed surface were dominated by a few low molecular mass proteins. Figure 17 (b) demonstrates that HP extraction and separation by SDS-PAGE is dependant on dithiothreitol (DTT).

Even though HP is an abundant seed constituent and a potent allergen, there have been no studies on the expression or localization of the protein or any description of the corresponding gene. This is the first report on the isolation and characterization of HP cDNA (SEQ ID NO:6) and the corresponding genomic clone (SEQ ID NO:7), the pattern of gene expression (Figure 10 (e)), and the localization of the protein (Figure 14 (b)) and its effect on seed luster (Figure 16). Figure 14 (c) shows that the presence of surface protein is related to the luster, or light reflective properties of the seed surface. Surface extracts from shiny seeded phenotypes usually contained far less protein than dull seeded extracts. Moreover, there were large differences in the amount of protein present on the seed surfaces of the two bloom phenotypes examined.

These results also show that the outermost components of the seed coat are in fact derived from the inner layer of the pod wall (see Figure 14 (a)).

The cDNA and genomic copies of the seed-coat associated HP gene were obtained from lambda libraries prepared from cultivar Harosoy 63. The genomic DNA sequence comprises a promoter region from 1-2526 of SEQ ID NO:7. Within this promoter region are located clustered direct repeats (between 1-586; see also Figure 11 (c). and a TATA box located at position 2442-2447. The ORF for HP is between 2526-2882, with the translational start site at 2526, followed by a signal sequence from 2526-2642, and the mature protein from 2643-2882. Also noted within the genomic sequence are six polyadenylation signals and a polyadenylation site at bp 3193.

Developmental and tissue specific expression patterns for the *HP* gene were determined by RNA blot analysis and *in situ* hybridization. Representative RNA blots, probed with *HP* cDNA, are shown in Figures 10 (e) and (f). These results show that *HP* is highly expressed in the pod during the mid to late stages of seed development. Hybridization signals were also observed in seed coat RNA samples. No expression was evident in the flower, leaf, embryo, stem, or root. We also compared *HP* transcript levels of two different seed luster phenotypes that differed in the amount of HP present on their seed surfaces. Figure 10 (g) shows that HP mRNA levels are several fold greater in dull seeded plants that accumulate large amounts of HP on the seed surface when compared to shiny seeded plants that have only trace amount of HP on the seed surface. Faint signals, corresponding to low *HP* transcript levels, were detectable in shiny seeded phenotypes after prolonged exposure times (not shown).

Localization of *HP* gene expression by *in situ* hybridization is shown in Figures 14 (b), (c) and (d). At six days post anthesis (DPA) expression of *HP* is limited to the membranous inner layer of the pericarp. By 12 DPA expression is very strong and the inner epidermis is showing signs of becoming detached from the rest of the pericarp and, in places, is adhering to the seed surface. Tissue sections from this stage of development also showed strong hybridization signals in the sclerenchyma, indicating that *HP* expression occurs throughout the endocarp. Portions of membranous endocarp adhere to the seed during the course of development (see Figures 14 (a) and 16) and thus constitute a newly identified component of the seed coat of mature, fully developed soybeans. The deposition of this material alters the physical properties and the composition of the seed surface, as shown by SDS-PAGE analysis (Figures 17 (a), (b) and (c)) and by scanning electron microscopy (Figure 16). A comparison of dull- and shiny-seeded cultivars reveals that the *HP* gene controls this phenotypic trait in soybeans.

In Figure 14 (b) can be seen the expression of a gene under the control of the HP promoter, The promoter (nucleotides 1-2526 of SEQ ID NO:7) is active within the membranous endocarp associated with the outer seed-coat.

SC 4, 20 and 21

Genes expressing specifically in seed coat tissue were isolated from a seed coat cDNA library obtained from seed coats in later stages of development.

SC4

The deduced protein sequence from the SC4 cDNA (Figure 19 (a); SEQ ID NO:3) consists of 289 amino acids and has a molecular mass of 31.9 kDa and a predicted pI of 7.95. Three puatative glycosylation sites are present at positions 92, 128 and 269. The putative polypeptide encoded by SC4 exhibits similarity with proteins that comprises a BURP domain (see Figure 19 (b)). The BURP domain is a long carboxyl terminal domain containing a number of highly conserved amino acids (Hattori J. et al., 1998. Mol. Gen. Genet. 259: 424-428). The genomic sequence of *sc*4 is provided in SEQ ID NO:9 (also see Restriction map Figure 11 (d)) and comprises a promoter from nucleotides 1-5514 of SEQ ID NO:9.

The expression of a gene under control of the SC4 promoter (nucleotides 1-5514 of SEQ ID NO:9) within soybean seed coat at 3 days after anthesis is shown in Figure 13 (a). The activity of the promoter is localized within the inner integument (arrow). Other areas of brightness in this figure include the recurrent vascular bundles in the funiculus, and the trichomes of the pod (the bright areas are due to the birefringence of crystalline areas in the cell walls, and are also present in the negative control; data not shown).

RNA samples from seed coat, embryo, stem, root, leaf, pod and flower were hybridized with a radiolabelled SC4 cDNA probe (Figure 10 (a)) to determine organ specificity of the expression of SC4. The sc4 transcript was only expressed in the seed coat organ. It was estimated that the size sc4 mRNA was 1.2 kb (data not shown).

Northern blot analysis was carried out to determine the temporal expression pattern of sc4. RNA from seed coat, embryo and pod organs between 6-24 dpa were hybridized with a radiolabelled SC4 cDNA probe. No gene expression was observed in any of the embryo development stages examined (Figure 20 (a)). sc4 expression was apparent in the seed by 6 dpa. After 6 dpa the expression of sc4 in the seed coat increased ~4-fold to its maximum detected level between 9-12 dpa. By 15 dpa sc4 expression had decreased by ~2.5-fold dpa and continued to decline to just detectable levels by 18 dpa (Fig. 3.7). Expression of sc4 could only be detected in the seed coat at 21-24 dpa when the filter was over-exposed. Gene expression of sc4 in the pod was detected from 12-21 dpa only after over-exposure of the filter (data not shown).

To analyse the distribution of sc4 expression with respect to cell differentiation during seed coat development *in situ* hybridization was performed on seed sections from 3-24 dpa seeds. sc4 was expressed throughout the inner integument of the seed coat at 3 dpa (Figures 13 (a) and 21). By 6 dpa the expression pattern of sc4 had changed, and was localized to the outer integument parenchyma but not to the vascular tissue embedded within this layer. sc4 expression in the outer integument was maintained until 18 dpa after which time no further expression was detected (see Table 4 in Examples). In concurrence with northern blot analysis, the *in situ* hybridization results revealed that sc4 expression increased to a maximum between 9-12 dpa and decreased thereafter (Table 4, in Examples). In addition, expression of sc4 was not observed in the embryo of seed at 3-6 dpa.

Expression of a gene under the control of the SC4 promoter (1-5514 of SEQ ID NO:9) is seen in Figures 13 (a) and 21.

Southern blot analysis was carried out to examine the gene family composition of sc4. Soybean genomic DNA was cleaved with Eco RI, Hind III and Xba I. which do not have recognition sites in the SC4c cDNA sequence. Under conditions of low to high stringency (i.e., from 40-10°C below Tm of the probe) the SC4 cDNA probe hybridized to a single band (Figure 22) and therefore sc4 appears to be a single gene.

Southern blot analysis was also performed to determine the occurrence of sc4 within the following plant species: pea (*Pisum sativum*), canola *(Brassica napus*), oat (*Avena sativa*), onion (*Allium cepa*), pepper (*Capsicum annuum*), Mimosa sp. (*Mimosa pudica*), black spruce (*Picea mariana* (Mill) B.S.P.), birch (*Betula pendula* Roth). The genomic DNA was digested with Eco RI. Under all stringency conditions it was observed that the radiolabelled SC4 cDNA probe hybridized to only soybean genomic DNA (Figure 22 (b)). Further analysis of more related species to soybean need to be carried out.

SC20

The open reading frame of SC20 encodes a putative protein of 770 amino acid residues with a calculated molecular mass of 82.688 kDa and a predicted pI of 6.93. The predicted protein has ten potential N-glycosylation sites (Figure 23 (b)). The hydropathy profile (Figure 23 (c)) of SC20 protein revealed that the first 23 amino acids constitute a hydrophobic region typical of an eukaryotic signal peptide. From northern blot analysis, the SC20 cDNA clone hybridizes to a ~2.5 kb transcript.

The genomic sc20 clone is 7235 bp in length (see Figure 23 (a) for restriction map, and SEQ ID NO:8). Alignment of sc20 genomic and SC20

cDNA sequences revealed that *sc*20 contained eight introns of 94 bp, 101 bp, 185 bp, 80 bp, 154 bp, 112 bp, 110 bp and 93 bp respectively (Figure 23 (a)). A search of the 5' upstream region of sc20 revealed three potential transcription start sites at positions 1085, 1156 and 2272. The promoter region of *sc*20 spans nucleotides 1-2450 of SEQ ID NO:8.

Sequence comparisons (Figure 23 (d)) revealed that the putative polypeptide encoded by SC20 was similar to plant proteins belonging to the Pyrolysin family in the clan of serine proteases known as the subtilases (Barrett A.J. and Rawlings N.D., 1995. Arch. Biochem. Biophys. 318:247-250; Siezen, R. J. and Leunissen, J. A. M. 1997. Protein Sci. 6: 501-523.). The SC20 protein comprises 3 domains: a signal peptide of 23 residues followed by a prosequence of 93 residues and a mature domain of 654 residues. The predicted mature domain of SC20 has a calculated molecular weight of 69.918 kDa and an isoelectric point of 6.34.

Northern blot analysis was carried out to determine specificity of sc20 expression in various soybean organs i.e., seed coat, embryo, stem, root, leaf, pod and flower (Figure 10 (b)). sc20 has seed coat-specific expression as its mRNA was detected only in the seed coat organ. The sc20 transcript was determined to be approximately 2.5 kb (data not shown). Even after prolonged exposure of the filter, no sc20 transcripts was detected in any of the other plant organs.

Northern blot analysis was performed to determine the temporal gene expression pattern of sc20 in seed coat, embryo and pod organs of soybean. Total RNA prepared from organs between 6- 24 dpa were probed with a radiolabelled SC20 cDNA probe. sc20 expression was detected at 9 dpa and rose 1.5 fold to its maximum observed level at 12 dpa (Figure 24). By 18 dpa accumulation of sc20 mRNA had decreased 4-fold. Prolonged exposure of the filter enabled detection of sc20 expression at 6 dpa and 21-24 dpa. No gene

expression was observed at any stage of embryo or pod development examined even after prolonged exposure of the filters. This confirmed that sc20 expression was seed coat-specific.

In situ hybridization was carried out to analyse the spatial gene expression pattern of sc20 within the seed coat between 3-24 dpa. Seed sections were hybridized with radiolabelled sense and anti-sense SC20 RNA probes. No birefringent cell structures were evident in the seed sections used (Figure 24).

Gene expression of sc20 was localized to the thick-walled parenchyma of the outer integument (see Figures 13 (b) and 24). The temporal expression pattern of 9-21 dpa expression with an observed peak at 12 dpa was almost identical to that determined by northern blot analysis (Table 4, in Examples). sc20 transcripts were not detected in the embryo between 3-6 dpa. The *in situ* hybridization results of the seed sections concur with the northern blot analysis that within the seed organ sc20 is expressed only in the seed coat organs.

Expression of gene under control of the SC20 promoter (1-245 of SEQ ID NO:8) is seen in Figures 13 (b) and 24.

Southern blot analysis was performed to ascertain whether *sc*20 is a single gene or a member of a gene family. Soybean genomic DNA was cleaved with Eco RI, Hind III, Xba I and Eco RV which have three, four, two and one recognition site(s) respectively in the sc20 clone (see Figure 23 (a)). Under conditions of high stringency to detect genes with at least 90% similarity to sc20 the probe hybridized to a single band (Figure. 25 (b)). Under medium stringency conditions to observe genes with 80% similarity to sc20 it was observed that the SC20 probe annealed to 2-3 bands for each digest (Figure 25 (a)). Under conditions of low stringency i.e., 40°C below Tm the SC20 probe hybridized to several more bands from each digest (data not shown). This suggested that sc20

is a member of a small gene family composed of 2-3 members and that the soybean genome contains several genes which are more distantly related to sc20.

Southern blot analysis was also performed to determine the distribution of sc20 among a number of diverse plant species i.e., pea (*Pisum sativum*), canola (*Brassica napus*), oat (*Avena sativa*), onion (*Allium cepa*), pepper *(Capsicum annuum*), Mimosa sp. (*Mimosa pudica*), black spruce (*Picea mariana* (Mill) B.S.P.), birch (*Betula pendula* Roth). The genomic DNA was restricted with Eco RI. The SC20 cDNA probe hybridized to only the genomic DNA of soybean (Figure 25 (c)) irrespective of stringency conditions utilized. It is possible that the gene may exist in more species more closely related to soybean.

SC21

The expression of a gene under the control of SC21 promoter (see Figure 11 (b)) within seed coat tissues at 15 days after anthesis is shown in Figure 13 (c). Note specific localization of the probe in the thin-walled parenchyma of the outer integument, including the area immediately surrounding the tracheid bar (arrow).

The nucleotide sequences of SC21 (SEQ ID NO:5) and SC17 were identical apart from the position of the poly (A) tail and were just less than 65% similar to a Cicer arientinum (chickpea) mRNA for an unknown protein.

The expression of genes under the control of seed-coat promoters of this invention are shown in Figures 10, 13, 14, 21 and 24.

The results of these and other experiments indicating the expression patterns of these genes is summarized in Table 4 within the Examples section.

The promoters of the present invention can be used to control the expression of any given gene spatially and developmentally within developing seed coats, or seed-coat associated tissues. Some examples of such uses, which are not to be considered limiting, include:

1. Modification of storage reserve yields in seed coats, such as starch by the expression of yeast invertase to mobilize the starch, or increasing starch levels by increasing the sink strength by enhancing carbon unloading into seeds, by expressing invertase in specific seed coat tissues, or reduce starch levels by inhibit starch biosynthesis through the expression of the antisense transcript of ADP-glucose pyrophosphorylase.

2. Modification of seed colour contributed by anthocyanin pigments or condensed tannins in the seed coats by expression of antisense transcripts of the phenylalanine ammonia lyase or chalcone synthase genes.

3. Modification of fibre content in seed-derived meal by expression of antisense transcripts of the caffeic acid-o-methyl transferase or cinnamoyl alcohol dehydrogenase genes.

4. Inhibition of seed coat maturation by expression of ribonuclease genes to allow for increased seed size, and to reduce the relative biomass of seed coats, and to aid in dehulling of seeds.

5. Expression of genes in seed coats coding for insecticidal proteins such as α-amylase inhibitor or protease inhibitor.

6. Partitioning of seed metabolites such as glucosinolates into seed coats for fungal or insect resistance.

7. Production of high value proteins in seed coats for use as pharmaceuticals or for use in industrial processes.

8. Control of seed borne diseases by expressing antifungal antiviral, or anti-bacterial proteins within the seed coat

Furthermore, modifications of the nucleotide, or amino acid, sequence of HP, or the preparation of chimeric gene constructs comprising the regulatory region of HP associated with a gene of interest will result in:

- alterations in the textural, visual, chemical or other properties of the seed coat, including the seed surface;

- the production of plants that are less susceptible to seed borne and pod diseases by expressing heterologous proteins in tissues of the ovary wall;

- lessening the health hazard of seed dust exposure by genetic selection or transformation, to produce plants with reduced allergenic protein expression on the seed surface

Thus this invention is directed to such promoter and gene combinations. Further this invention is directed to such promoter and gene combinations in a cloning vector, wherein the gene is under the control of a seed coat specific promoter and is capable of being expressed in a plant cell transformed with the vector. This invention further relates to transformed plant cells and transgenic plants regenerated from such plant cells. The promoter and promoter gene combination of the present invention can be used to transform any plant cell for the production of any transgenic plant. The present invention is not limited to any plant species.

The following list summarises the nucleotide sequence data in the SEQUENCE LISTING of the present application:

pT218 genomic DNA sequence is found in SEQ ID NO:1;

Ep genomic DNA sequence is listed in SEQ ID NO:2;

SC4 cDNA sequence is presented in SEQ ID NO:3;

SC20 cDNA sequence is in SEQ ID NO:4;

SC21 cDNA sequence is presented in SEQ ID NO:5;

HP cDNA is listed in SEQ ID NO:6;

HP genomic DNA sequence is found in SEQ ID NO:7;

SC20 genomic DNA sequence is listed in SEQ ID NO:8; and

SC4 genomic DNA sequence is presented in SEQ ID NO:9.

While this invention is described in detail with particular reference to preferred embodiments thereof, said embodiments are offered to illustrate but not limit the invention.

EXAMPLES

Characterization of a Seed Coat-Specific GUS Fusion

Transfer of binary constructs to *Agrobacterium* and leaf disc transformation of *Nicotiana tabacum* SR1 were performed as described by Fobert *et al.* (1991, *Plant Mol. Biol.* **17**, 837-851). Plant tissue was maintained on 100 μg/ml kanamycin sulfate (Sigma) throughout *in vitro* culture.

Nine-hundred and forty transgenic plants were produced. Several hundred independent transformants were screened for GUS activity in developing seeds using the fluorogenic assay. One of these, T218, was chosen for detailed study because of its unique pattern of GUS expression.

Fluorogenic and histological GUS assays were performed according to Jefferson (*Plant Mol. Biol. Rep.*, 1987, 5, 387-405), as modified by Fobert *et al.* (*Plant Mol. Biol.*, 1991, 17, 837-851). For initial screening, leaves were harvested from *in vitro* grown plantlets. Later flowers corresponding to developmental stages 4 and 5 of Koltunow *et al.* (*Plant Cell*, 1990, 2, 1201-1224) and beige seeds, approximately 12-16 dpa (Chen *et al.*, 1988, *EMBO J.* 7, 297-302), were collected from plants grown in the greenhouse. For detailed, quantitative analysis of GUS activity, leaf, stem and root tissues were collected from kanamycin resistant F1 progeny of the different transgenic lines grown *in vitro*. Floral tissues were harvested at developmental stages 8-10 (Koltunow *et al.*, 1990, *Plant Cell* 2, 1201-1224) from the original transgenic plants. Flowers of these plants were also tagged and developing seeds were collected from capsules at 10 and 20 dpa. In all cases, tissue was weighed, immediately frozen in liquid nitrogen, and stored at -80°C.

Tissues analyzed by histological assay were at the same developmental stages as those listed above. Different hand-cut sections were analyzed for each organ. For each plant, histological assays were performed on at least two different occasions to ensure reproducibility. Except for floral organs, all tissues were assayed in phosphate buffer according to Jefferson (1987, *Plant Mol. Biol. Rep.* 5, 387-405), with 1 mM X-Gluc (Sigma) as substrate. Flowers were assayed in the same buffer containing 20% (v/v) methanol (Kosugi *et al.*, 1990, *Plant Sci.* 70, 133-140).

Tissue-specific patterns of GUS expression were only found in seeds. For instance, GUS activity in plant T218 (Figure 1) was localized in seeds from 9 to 17 days postanthesis (dpa). GUS activity was not detected in seeds at other stages of development or in any other tissue analyzed which included leaf, stem, root, anther, ovary, petal and sepal (Figure 1). Histological staining with X-Gluc revealed that GUS expression in seeds at 14 dpa was localized in seed coats

but was absent from the embryo, endosperm, vegetative organs and floral organs (results not shown).

The seed coat-specificity of GUS expression was confirmed with the more sensitive fluorogenic assay of seeds derived from reciprocal crosses with untransformed plants. The seed coat differentiates from maternal tissues called the integuments which do not participate in double fertilization (Esau, 1977, *Anatomy of Seed Plants*. New York: John Wiley and Sons). If GUS activity is strictly regulated, it must originate from GUS fusions transmitted to seeds maternally and not by pollen. As shown in Table 3, this is indeed the case. As a control, GUS fusions expressed in embryo and endosperm, which are the products of double fertilization, should be transmitted through both gametes. This is illustrated in Table 3 for GUS expression driven by the napin promoter (BngNAPI, Baszczynki and Fallis, 1990, *Plant Mol. Biol.* **14,** 633-635) which is active in both embryo and endosperm (data not shown).

Table 3. GUS activity in seeds at 14 days post anthesis.

Cross		GUS Activity
♀	♂	nmole MU/min/mg Protein
T218	T218	1.09 ± 0.39
T218	WT[a]	3.02 ± 0.19
WT	T218	0.04 ± 0.005
WT	WT	0.04 ± 0.005
NAP-5[b]	NAP-5	14.6 ± 7.9
NAP-5	WT	3.42 ± 1.60
WT	NAP-5	2.91 ± 1.97

[a] WT, untransformed plants

[b] Transgenic tobacco plants with the GUS gene fused to the napin, BngNAP1, promoter (Baszczynski and Fallis, 1990, *Plant Mol. Biol.* **14**, 633-635).

Cloning and Analysis of the Seed Coat-Specific GUS Fusion

Genomic DNA was isolated from freeze-dried leaves using the protocol of Sanders *et al.* (1987, *Nucleic Acid Res.* **15**, 1543-1558). Ten micrograms of T218 DNA was digested for several hours with *Eco*RI using the appropriate manufacturer-supplied buffer supplemented with 2.5 mM spermidine. After electrophoresis through a 0.8% TAE agarose gel, the DNA size fraction around 4-6 kb was isolated, purified using the GeneClean kit (BIO 101 Inc., LaJolla,

CA), ligated to phosphatase-treated *Eco*RI-digested Lambda GEM-2 arms (Promega) and packaged *in vitro* as suggested by the supplier. Approximately 125,000 plaques were transferred to nylon filters (Nytran, Schleicher and Schuell) and screened by plaque hybridization (Rutledge *et al.*, 1991, *Mol. Gen. Genet.* **229**, 31-40), using the 3' (termination signal) of the *nos* gene as probe (probe #1, Figure 2). This sequence, contained in a 260 bp *Sst*I/*Eco*RI restriction fragment from pPRF-101 (Fobert *et al.*, 1991, *Plant Mol. Biol.* **17**, 837-851), was labelled with [α-^{32}P]-dCTP (NEN) using random priming (Stratagene). After plaque purification, phage DNA was isolated (Sambrook *et al.*, 1989, A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press), mapped and subcloned into pGEM-4Z (Promega). The *Eco*RI fragment and deletions shown in Figure 2 were inserted into pBIN19 (Bevan, 1984, *Nucl. Acid Res.* **12**, 8711-8721). Restriction mapping was used to determine the orientation of the fusion in pBIN19 and to confirm plasmid integrity. Plants were transformed with a derivative which contained the 5' end of the GUS gene distal to the left border repeat. This orientation is the same as that of the GUS gene in the binary vector pBl101 (Jefferson, 1987, *Plant Mol. Biol. Rep.* **5**, 387-405).

The GUS fusion in plant T218 was isolated as a 4.7 kb *Eco*RI fragment containing the 2.2kb promoterless GUS-*nos* gene at the T-DNA border of pPRF120 and 2.5 kb of 5' flanking tobacco DNA (pT218, Figure 2), using the *nos* 3' fragment as probe (probe #1, Figure 2). To confirm the ability of the flanking DNA to activate the GUS coding region, the entire 4.7 kb fragment was inserted into the binary transformation vector pBIN19 (Bevan, 1984, *Nucl. Acid Res.* **12**, 8711-8721), as shown in Figure 2. Several transgenic plants were produced by *Agrobacterium*-mediated transformation of leaf discs. Southern blots indicated that each plant contained 1-4 T-DNA insertions at unique sites. The spatial patterns of GUS activity were identical to that of plant T218. Histologically, GUS staining was restricted to the seed coats of 14 dpa seeds and was absent in embryos and 20 dpa seeds (results not shown). Fluorogenic assays

of GUS activity in developing seeds showed that expression was restricted to seeds between 10 and 17 dpa, reaching a maximum at 12 dpa (Figure 3 (a) and 3 (b)). The 4.7 kb fragment therefore contained all of the elements required for the tissue-specific and developmental regulation of GUS expression.

To locate regions within the flanking plant DNA responsible for seed coat-specificity, truncated derivatives of the GUS fusion were generated (Figure 2) and introduced into tobacco plants. Deletion of the region approximately between 2.5 and 1.0 kb, 5' of the insertion site (pT218-2, Figure 2) did not alter expression compared with the entire 4.7 kb GUS fusion (Figures 3b and 4). Further deletion of the DNA, to the *Sna*BI restriction site approximately 0.5 kb, 5' of the insertion site (pT218-3, Figure 2), resulted in the complete loss of GUS activity in developing seeds (Figures 3b and 4). This suggests that the region approximately between 1.0 and 0.5 kb, 5' of the insertion site contains elements essential to gene activation. GUS activity in seeds remained absent with more extensive deletion of plant DNA (pT218-4, Figures 2, 3b and 4) and was not found in other organs including leaf, stem, root, anther, petal, ovary or sepal from plants transformed with any of the vectors (data not shown).

The transcriptional start site for the GUS gene in plant T218 was determined by RNase protection assays with RNA probe #4 (Figure 2) which spans the T-DNA/plant DNA junction. For RNase protection assays, various restriction fragments from pIS-1, pIS-2 and pT218 were subcloned into the transcription vector pGEM-4Z as shown in Figures 7 and 2, respectively. A 440bp *Hin*dIII fragment of the tobacco acetohydroxyacid synthase *SURA* gene was used to detect *SURA* and *SURB* mRNA. DNA templates were linearized and transcribed *in vitro* with either T7 or SP6 polymerases to generate strand-specific RNA probes using the Promega transcription kit and [α-^{32}P]CTP as labelled nucleotide. RNA probes were further processed as described in Ouellet *et al.* (1992, *Plant J.* **2**, 321-330). RNase protection assays were performed as described in Ouellet *et al.*, (1992, *Plant J.* **2**, 321-330), using 10-30 μg of total

RNA per assay. Probe digestion was done at 30°C for 15 min using 30 μg ml^{-1} RNase A (Boehringer Mannheim) and 100 units ml^{-1} RNase T1 (Boehringer Mannheim). Figure 5 shows that two termini were mapped in the plant DNA. The major 5' terminus is situated at an adenine residue, 122 bp upstream of the T-DNA insertion site (Figure 6). The sequence at this transcriptional start site is similar to the consensus sequence for plant genes (C/TTC↓ATCA; Joshi, 1987 *Nucleic Acids Res.* **15**, 6643-6653). A TATA box consensus sequence is present 37 bp upstream of this start site (Figure 6). The second, minor terminus mapped 254 bp from the insertion site in an area where no obvious consensus motifs could be identified (Figure 6).

The tobacco DNA upstream of the insertion site is very AT-rich (>75%, see Figure 7). A search for promoter-like motifs and scaffold attachment regions (SAR), which are often associated with promoters (Breyne *et al.*, 1992, *Plant Cell* **4**, 463-471; Gasser and Laemmli, 1986, *Cell* **46**, 521-530), identified several putative regulatory elements in the first 1.0 kb of tobacco DNA flanking the promoterless GUS gene (data not shown). However, the functional significance of these sequences remains to be determined.

Cloning and Analysis of the Insertion Site from Untransformed Plants

A lambda DASH genomic library was prepared from DNA of untransformed *N. tabacum* SR1 plants by Stratagene for cloning of the insertion site corresponding to the gene fusion in plant T218. The screening of 500,000 plaques with probe #2 (Figure 2) yielded a single lambda clone. The *Eco*RI and *Xba*I fragments were subcloned in pGEM-4Z to generate pIS-1 and pIS-2. Figure 7 shows these two overlapping subclones, pIS-1 (3.0 kb) and pIS-2 (1.1 kb), which contain tobacco DNA spanning the insertion site (marked with a vertical arrow). DNA sequence analysis (using dideoxy nucleotides in both directions) revealed that the clones, pT218 and pIS-1, were identical over a

length of more than 2.5 kb, from the insertion site to their 5' ends, except for a 12 bp filler DNA insert of unknown origin at the T-DNA border (Figure 6 and data not shown). The presence of filler DNA is a common feature of T-DNA/plant DNA junctions (Gheysen *et al.*, 1991, *Gene* 94, 155-163). Gross rearrangements that sometimes accompany T-DNA insertions (Gheysen *et al.*, 1990, *Gene* 94, 155-163; and 1991, *Genes Dev.* 5, 287-297) were not found (Figure 6) and therefore could not account for the promoter activity associated with this region. The region of pIS-1 and pIS-2, 3' of the insertion site is also very AT-rich (Figure 7).

To determine whether there was a gene associated with the pT218 promoter, more than 3.3 kb of sequence contained with pIS-1 and pIS-2 was analyzed for the presence of long open reading frames (ORFs). However, none were detected in this region (data not shown). To determine whether the region surrounding the insertion site was transcribed in untransformed plants, Northern blots were performed with RNA from leaf, stem, root, flower and seeds at 4, 8, 12, 14, 16, 20 and 24 dpa. Total RNA from leaves was isolated as described in Ouellet *et al.*, (1992, *Plant J.* **2**, 321-330). To isolate total RNA from developing seeds, 0.5 g of frozen tissue was pulverized by grinding with dry ice using a mortar and pestle. The powder was homogenized in a 50 ml conical tube containing 5 ml of buffer (1 M Tris HCl, pH 9.0, 1% SDS) using a Polytron homogenizer. After two extractions with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1), nucleic acids were collected by ethanol precipitation and resuspended in water. The RNA was precipitated overnight in 2M LiCl at 0°C, collected by centrifugation, washed in 70% ethanol and resuspended in water. Northern blot hybridization was performed as described in Gottlob-McHugh *et al.* (1992, *Plant Physiol.* **100**, 820-825). Probe #3 (Figure 2) which spans the entire region of pT218 5' of the insertion did not detect hybridizing RNA bands (data not shown). To extend the sensitivity of RNA detection and to include the region 3' of the insertion site within the analysis, RNase protection assays were performed with 10 different RNA probes

that spanned both strands of pIS-1 and pIS-2 (Figure 7). Even after lengthy exposures, protected fragments could not be detected with RNA from 8, 10, 12 dpa seeds or leaves of untransformed plants (see Figure 5 for examples with two of the probes tested). The specific conditions used allowed the resolution of protected RNA fragments as small as 10 bases (data not shown). Failure to detect protected fragments was not due to problems of RNA quality, as control experiments using the same samples detected acetohydroxyacid synthase (*AHAS*) *SURA* and *SURB* mRNA which are expressed at relatively low abundance (data not shown). Conditions used in the present work were estimated to be sensitive enough to detect low-abundance messages representing 0.001-0.01% of total mRNA levels (Ouellet *et al.*, 1992, *Plant J.* **2**, 321-330). Therefore, the region flanking the site of T-DNA insertion does not appear to be transcribed in untransformed plants.

Genomic Origins of the Insertion Site

Southern blots were performed to determine if the insertion site is conserved among *Nicotiana* species. Genomic DNA (5 μg) was isolated, digested and separated by agarose gel electrophoresis as described above. After capillary transfer on to nylon filters, DNA was hybridized, and probes were labelled, essentially as described in Rutledge *et al.* (1991, *Mol. Gen. Genet.* **229**, 31-40). High-stringency washes were in 0.2 x SSC at 65°C while low-stringency washes were in 2 x SSC at room temperature. In Figure 8, DNA of the allotetraploid species *N. tabacum* and the presumptive progenitor diploid species *N. tomentosiformis* and *N. sylvestris* (Okamuro and Goldberg, 1985, *Mol. Gen. Genet.*, **198**, 290-298) were hybridized with probe #2 (Figure 2). Single hybridizing fragments of identical size were detected in *N. tabacum* and *N. tomentosiformis* DNA digested with *Hin*dIII, *Xba*I and *Eco*RI, but not in *N. sylvestris*. Hybridizations with pIS-2 (Figure 8) which spans the same region but includes DNA 3' of the insertion site yielded the same results. They did not reveal hybridizing bands, even under conditions of reduced stringency, in

additional *Nicotiana* species including *N. rustica*, *N. glutinosa*, *N. megalosiphon* and *N. debneyi* (data not shown). Probe #3 (Figure 2) revealed the presence of moderately repetitive DNA specific to the *N. tomentosiformis* genome (data not shown). These results suggest that the region flanking the insertion site is unique to the *N. tomentosiformis* genome and is not conserved among related species as might be expected for regions that encode essential genes.

Cloning of seed-coat genes from Soybean:

a) Isolation of seed-coat cDNA clones

A seed coat cDNA library was constructed in Lambda GEM-4 from poly(A)$^+$ mRNA isolated from soybean [*Glycine max* (L.) Merrill] seed coats. A sample of the total amplified library was used to sub-clone inserts from the original lambda vector into pBK-CMV (Stratagene). Random clones were selected from this mass excision for plasmid purification and single-run DNA sequencing to construct an expressed sequence tag (EST) database.

For differential screening, an additional cDNA library was constructed from cultivar Maple Presto (*EpEp*) seed coats. The seed coats were harvested from seeds of four fresh weight groups: <50 mg, 50-100 mg, 150-250 mg and >250 mg, to represent all developmental stages. Total RNA was isolated from the seed coats using Trizole reagent (BRL) from which poly (A)+ RNA was isolated using Oligotex resin (Qiagen). First and second strand cDNAs were synthesized using the Riboclone cDNA synthesis kit and then cloned into a lambda GEM-4 vector (Promega). This seed coat library was differentially screened with positive and negative cDNA probes to identify genes preferentially expressed in the seed coat. The positive probe was derived from poly (A)+ mRNA isolated from seed coat tissues while the negative probe was made from poly (A)+ mRNA from seedling, flower bud, leaf, pod and root tissue. The cDNA library was screened with cDNA synthesized from RNA using oligo$(dT)_{15}$

primer, and hybridizations were carried out in Denhardt's solution (Sambrook et al. (1989) Molecular Cloning, Second Edition) at 65°C; wash 4 x 30 minutes 0.1X SSC 0.1% SDS at 65°C.

Twenty-one positive clones were identified after plaque purification. The Lambda vector GEM-4 contains a complete pGEM1 plasmid. During the cloning procedure the cDNA is inserted into the Lambda vector at the multicloning site of this plasmid. The entire pGEM1 plasmid, containing the cDNA insert, can be removed from the Lambda vector by digestion with *Spe1* and then can be relegated to form a functional plasmid. Except for SC11 and SC19, the insert was removed from pGEM1 by digestion with *Xba*1 and *EcoR*1 and ligated into an alternative plasmid vector pGEM4-Z. Following this protocol 21 seed coat clones were used to transform *E. coli* DH5α. No transformants were obtained with seed coat clones SC7 and SC10 and so these clones were not processed further.

Seed surface proteins were obtained from soybean. A single seed was placed in a 2 mL plastic capped test tube and surface proteins were extracted by adding 0.5 mL of a buffer-detergent solution containing 10 mM Tris-Cl (pH 7.5) 0.5% SDS, and 20 mM DTT, and placing the tube in a boiling water bath for 2 min. The contents of the tube were mixed and an aliquot was withdrawn and centrifuged for 5 min at 14,000 g. Freshly prepared loading buffer containing 20 mM DTT was added to the sample and proteins were electrophoretically separated on 15% acrylamide gels in the presence of SDS (see Figure 17) using a modified Laemmli system, as described by Fling and Gregerson (1986, Anal Biochem 155:83-88). Fixation and visualization of the proteins by silver staining followed the method of Blum et al., (1987 Electrophoresis 8: 93-99). The amino terminal of the major peptides (indicated as HPS in Figure 17 (b)) were micro-sequenced from the blotted proteins according to Moos et al., (1988 J. Biol. Chem 263: 6005-6008). The resulting amino acid sequences were identical and matched existing sequences in the GenBank protein database for HP (Odani et

al., 1987 Eur J. Biochem 162, 485-491). Both peptides had alternative N-terminal residues of Ala or Ile, as has been previously noted for HP. The different electrophoretic mobilities of the two peptides could not be accounted for from the microsequencing analysis, but may be due to differences in glycosylation.

Several different soybean varieties were also compared by SDS-PAGE analysis (see Figure 17 (c)).

To obtain the cDNA transcript of HP, sequences in the seed coat expressed sequence tag database were searched for reading frames corresponding to the HP amino acid sequence. Using this strategy, several identical cDNA transcripts were isolated from the cDNA library obtained from Harosoy 63 seeds described above that included in their reading frames peptide sequences exactly matching HP. The encoded products of these DNA sequences were identified using the BLASTX program at the NCBI site.

b) Characterization of cDNA clones

Sequence analysis

Agarose gel electrophoresis of *Xba1/EcoR1* digests of the 19 remaining plasmid clones indicated that the inserts ranged in size from approximately 350bp to 1600bp, including the poly A tail. Inserts of the seed-coat clones were characterized by double stranded dideoxy sequencing of the 5' and 3' ends of the clones. A preliminary classification of the seed coat cDNA clones was made on the basis of sequence homology in the 3' and 5' ends of the clones. Based on sequence similarity with each other these 19 clones were grouped into 7 groups of clones. Sequence similarity was found between four of these groups and GenBank (with proline rich protein and three peroxidase groups). The three remaining groups had no sequence similarity with GenBank. SC4, SEQ ID

NO:3 (found to be the same as SC1), SC20, SEQ ID NO:4 (found to be the same as SC15), and SC21, SEQ ID NO:5, each represent one clone of each of the three groups which did not exhibit similarity with anything in the GenBank database.

SC4

The 1119 bp nucleotide sequence of SC4 (SEQ ID 3, Figure 19 (a); also see Restriction Map Figure 11 (d)) does not represent the full-length cDNA clone as it does not contain an ATG codon for translation initiation. Two typical polyadenylation signals (AATAAA) are located at positions 1096 and 1102. The deduced protein sequence from the SC4 cDNA (Figure 19 (a)) consists of 289 amino acids and has a molecular mass of 31.9 kDa and a predicted pI of 7.95. Three puatative glycosylation sites are present at positions 92, 128 and 269.

The putative polypeptide encoded by SC4 exhibits similarity with proteins that comprises a BURP domain (e.g. RD22, an *Arabidopsis thaliana* dehydration-responsive protein (Yamaguchi-Shinozaki K. and Shinozaki, K. 1993. Mol. Gen. Genet. 238: 17-25); PG1β, a *Lycopersicon esculentum* polygalacturonase isoenzyme 1 β subunit (Zheng L. et al., 1992. Plant Cell. 4: 1147-1156); Sali3-2, a *Glycine max* L aluminium-induced protein (Ragland M. and Soliman, K.M. 1997. Plant Physiol. 114: 395); USP, a *Vicia faba* unknown seed protein (Baumlein H. et al., 1991. Mol. Gen. Genet. 225: 459-467) and ADR6, a *Glycine max* L auxin-induced protein (Datta N. et al., 1993. Plant Mol. Biol. 21: 859-869); see Figure 19 (b)). The BURP domain is a long carboxyl terminal domain containing a number of highly conserved amino acids (Hattori J. et al., 1998. Mol. Gen. Genet. 259: 424-428). The carboxyl terminal of the conceptual SC4 protein sequence contains the following conserved amino acids which are typical of the BURP domain proteins: two phenylalanine residues, two cysteine residues and four cysteine-histidine motifs which are also in the conserved alignment of CHX10CHX25-27CHX25-26 CH, where X is any amino

acid (Figure 19 (b)). This BURP domain proteins also share a similar structural make-up of 3-4 domains (Figure 19 (c)) i.e., an amino-terminal domain containing a hydrophobic sequence, a second domain which may or may not be conserved, a third domain consisting of tandem repeats of a short amino acid sequence (not all BURP domain proteins have this domain) and a long carboxyl-terminal BURP domain (Hattori J. et al.,1998. Mol. Gen. Genet. 259: 424-428). The tandem repeats which make up the third domain do not appear to have a common concensus sequence between the different BURP domain proteins. In addition to the BURP domain, the putative SC4 protein shares sequence similarity between its amino terminus and the conserved segment of the second domain possessed by several of the BURP domain proteins (Figure 19 (b)). It was also determined that the SC4 protein has a region containing two copies of the repeated sequence ESRSIXXYAG where X is any amino acid (Figure 19 (a)) which is similar to the structural organization of the third domain of several BURP domain proteins. Due to the extent of structural and sequence similarity between the SC4 protein and the BURP domain proteins it is likely that SC4 also contains a hydrophobic amino terminal if it was full-length.

SC20

The SC20 cDNA clone was sequenced (Figure. 23 (b)) and found to consist of 2447 bp with one 2310 bp open reading frame starting at nucleotide position 13 and ending at 2322. The TAG stop codon may be leaky as plants have tRNAs capable of misreading it. However, any readthrough will be terminated by a second stop codon TGA which is immediately adjacent to UAG. The 3' untranslated region contains one putative polyadenylation signal (AATAAA) located 21 nt after the stop codon.

The open reading frame of SC20 encodes a putative protein of 770 amino acid residues with a calculated molecular mass of 82.688 kDa and a predicted pI of 6.93. The predicted protein has ten potential N-glycosylation sites (Figure 23

(b)). The hydropathy profile (Figure 23 (c)) of SC20 protein revealed that the first 23 amino acids constitute a hydrophobic region typical of an eukaryotic signal peptide. From northern blot analysis, the SC20 cDNA clone hybridizes to a ~2.5 kb transcript (data not shown). SC20 was used to obtain the genomic clone which was from a soybean cv. Harovinton genomic library.

Sequence comparisons (Figure 23 (d)) revealed that the putative polypeptide encoded by SC20 was similar to a *Picea abies* (black spruce) AF70 protein (Sabala et al., 1997. Physiol. Plant. 99: 316-322); cucumisin, (Yamagata Y. et al. 1994 J. Biol. Chem. 269: 32725-32731) from *Cucumis melo* L. (musk melon); a pathogen-induced protein, P69B, (Tornero P. et al., 1997 J. Biol. Chem. 272: 14412-12219) from *Lycopersicon esculentum* (tomato) and a nodule-specific protein, Ag12, (Ribeiro A. et al., 1995 Plant Cell. 7: 785-794) from *Alnus glutinosa* (black alder). These plant proteins belong to the Pyrolysin family in the clan of serine proteases known as the subtilases (Barrett A.J. and Rawlings N.D., 1995. Arch. Biochem. Biophys. 318:247-250; Siezen, R. J. and Leunissen, J. A. M. 1997. Protein Sci. 6: 501-523.). The SC20 protein contains the conserved catalytic residues aspartate, histidine and serine as well as the highly conserved asparagine residue which is involved in stabilizing substrate binding. Moreover, the order of these four conserved residues in the SC20 protein is also a characteristic feature of subtilases. The SC20 protein also has a large sequence insertion between the conserved asparagine and serine residues found in plant subtilases but not in other subtilase members such as subtilisin BPN'(Power S.D. et al., 1986 PNAS. 83:3096-3100), kex2 (Mizuno K. et al., 1988 Biochem. Biophys. Res. Comm. 156: 246-254) or furin (van de Ven W.J.M. et al.,1990 Mol. Biol. Rep. 14: 265-275). Based on sequence similarity between the N-termini of mature plant subtilases and the SC20 protein (data not shown) it was predicted that the SC20 protein had a mature domain starting from residue 117. Therefore the SC20 protein appears to be composed of 3 domains: a signal peptide of 23 residues followed by a prosequence of 93 residues and a

mature domain of 654 residues. The predicted mature domain of SC20 has a calculated molecular weight of 69.918 kDa and an isoelectric point of 6.34.

HP

The cDNA sequence for HP (pHPScDNA1) is (SEQ ID NO:6) shown in Figure 15. The 700 bp transcript includes 30 bp of 5' untranslated region (UTR), an open reading frame (ORF) of 119 amino acids, and 313 bp of 3' UTR. Several polyadenylation signals were identified in the 3' UTR. The final 80 residues of deduced amino acid sequence exactly match the peptide sequence reported for the hydrophobic protein (Odani *et al.*, 1987, *Eur J Biochem* 162, 485-491). Thus, the HP cDNA transcript indicates that hydrophobic protein is translated with a leader sequence of 39 amino acids.

Northern blot analysis

Northern analysis, using the cDNA inserts of each clone as a probe, was performed to investigate the expression pattern of the 19 seed coat clones.

RNA isolation from leaf, stem, pod and flower tissue was optimized based on a protocol adapted from Tripure Isolation reagent kit (Boehringer Mannheim). Plant tissue was frozen in liquid nitrogen and homogenized with the Tripure reagent (a monophasic solution of phenol and guanidine thiocyanate). After the addition of chloroform the sample is centrifuged so that it separates into three phases. RNA is recovered from the upper aqueous phase by isopropanol precipitation. Due to the problem of polysaccharide contamination which increases with seed maturity, the isopropanol precipitation step was carried out in the presence of high salt which effectively maintains the polysaccharides in a soluble form whilst the RNA is precipitated.

Total RNA from seed-coat, embryo and root tissue was isolated as described by Fobert et al. (Plant J. 1994 6:567-577). Plant tissue was frozen in liquid nitrogen and homogenized in 1M Tris-HCl, pH9, 1% SDS buffer. The sample was extracted twice with equal volume phenol:chloroform:isoamyl alcohol (25:24:1), nucleic acids were collected by ethanol precipitation, collected by ethanol precipitation and resuspended in water. The RNA was precipitated overnight in 2M LiCl at 0°C, collected by centrifugation and resuspended in water.

RNA was denatured and size fractionated by formaldehyde gel electrophoresis and transferred onto nylon filters. Northern hybridization was carried out using radioactively labeled cDNA probes with hybridization in modified Church's buffer (Church and Gilbert (1994) PNAS USA 81: 1991-1995) at 65°C; wash 2 x 30 minutes 0.1X SSC 0.1% SDS at 65°C. From this analysis, it was observed that SC4, and SC20 have seed coat specific expression. *Ep* locus peroxidase has preferential expression within seed-coat tissues, and SC21 was only expressed in seed coat, stem, root and flower tissues. The results are shown in Figure 10 (a) - (d).

HP

For the analysis of HP expression, total RNA was isolated from roots, stems, leaves, flowers, pods, seed coats, and embryos dissected from soybean plants at various stages of development, according to published methods (Wang and Vodkin (1994) *Plant Mol Biol Rep* 12, 132-145). The RNA samples were quantitated by measuring absorbence at 260 nm, and by electrophoretic separation in formaldehyde gels and comparison to known standards. Samples of total RNA (10 μg each) were blotted to nylon membrane using a vacuum manifold apparatus and fixed by UV cross-linking. Digoxigenin-labelled cDNA was prepared according to instructions of the manufacturer (Boehringer) and used to probe the RNA blots. Results, Figures 10 (e) and (f) show that the *HP* gene is

highly expressed in the pod tissues during the later stages of development. Hybridization signals were also noted in RNA samples derived from seed coat tissue, but not in RNA samples from the leaf, flower, embryo, stem or root. These results, together with the data from the *in situ* hybridizations (see below) and the scanning electron microscopy analysis, indicate the *HP* gene is specifically expressed in the endocarp of the ovary wall. Pieces of this tissue detach from the pod wall and adhere to the seed surface during development, thus becoming a component of the mature seed coat (see Figure 14 (a).

SC4

RNA samples from seed coat, embryo, stem, root, leaf, pod and flower were hybridized with a radiolabelled SC4 cDNA probe (Figure 10 (a)) to determine organ specificity of the expression of SC4. The sc4 transcript was only expressed in the seed coat organ. It was estimated that the size sc4 mRNA was 1.2 kb (data not shown).

Northern blot analysis was carried out to determine the temporal expression pattern of sc4. RNA from seed coat, embryo and pod organs between 6-24 dpa were hybridized with a radiolabelled SC4 cDNA probe. At 6 dpa the seed is too small to separate the seed coat and embryo organs and so total RNA was isolated from an entire seed. sc4 expression was already apparent in the seed by 6 dpa. No gene expression was observed in any of the embryo development stages examined (Figure 20 (a)). sc4 mRNA transcripts were not observed in the embryo of 3-6 dpa seed sections examined by *in situ* hybridization using a radiolabelled SC4 antisense RNA probe (data not shown). Therefore the sc4 expression observed at 6 dpa in the seed tissue is likely to be seed coat derived. After 6 dpa the expression of sc4 in the seed coat increased ~4-fold to its maximum detected level between 9-12 dpa. By 15 dpa sc4 expression had decreased by ~2.5-fold dpa and continued to decline to just detectable levels by 18 dpa (Fig. 3.7). Expression of sc4 could only be detected

in the seed coat at 21-24 dpa when the filter was over-exposed. Gene expression of sc4 in the pod was detected from 12-21 dpa only after over-exposure of the filter (data not shown).

SC20

Northern blot analysis was carried out to determine specificity of sc20 expression in various soybean organs i.e., seed coat, embryo, stem, root, leaf, pod and flower (Figure 10 (b)). sc20 has seed coat-specific expression as its mRNA was detected only in the seed coat organ. The sc20 transcript was determined to be approximately 2.5 kb (data not shown). Even after prolonged exposure of the filter, no sc20 transcripts was detected in any of the other plant organs.

Northern blot analysis was performed to determine the temporal gene expression pattern of sc20 in seed coat, embryo and pod organs of soybean. Total RNA prepared from organs between 6- 24 dpa were probed with a radiolabelled SC20 cDNA probe. sc20 expression was detected at 9 dpa and rose 1.5 fold to its maximum observed level at 12 dpa (Figure 24). By 18 dpa accumulation of sc20 mRNA had decreased 4-fold. Prolonged exposure of the filter enabled detection of sc20 expression at 6 dpa and 21-24 dpa. No gene expression was observed at any stage of embryo or pod development examined even after prolonged exposure of the filters. This confirmed that sc20 expression was seed coat-specific.

b) In situ hybridization

To analyze the distribution of the clones mRNA expression with respect to cell differentiation during development, *in situ* hybridization, on sections from 3, 6, 9, 12, 15, 18, 21 and 24 DAF seeds was used. Seeds or parts of seeds were fixed in FAA fixative (50% ethanol, 5% acetic acid and 3.7% formaldehyde),

dehydrated in an ethanol/ tertiary butyl alcohol series and infiltrated and embedded in paraplast plus. Sections (5-10μm) were cut on a microtome, transferred onto Superfrost slides which are positively charged to allow better adherence of the sections to the slide surface. Prior to *in situ* hybridization the samples were dewaxed in a xylene/ethanol series. *In situ* hybridization was carried out with ^{35}S-labelled cDNA sense and anti-sense probes following the method of Cox and Goldberg (1998).

Ep

For the *in situ* analysis of *Ep* expression, flowers were tagged on days of full anthesis when the banner petal was fully extended and harvested at three day intervals from 1-30 days post anthesis (DPA), and at 45 DPA [19]. Tissue samples were fixed in a solution of 3.7% formaldehyde, 50% ethanol, and 5% acetic acid for 3 h at room temperature, dehydrated in an ethanol series (50, 60, 70, 80, 90, 95, 100%) then infiltrated with t-butyl alcohol (TBA) in ethanol in a stepwise series (25, 50, 75, and 100%), followed by infiltration with Paraplast and several changes of pure melted Paraplast at 57 °C. After infiltration, samples were placed in blocks and allowed to harden. Sections of 8-10 :m were cut on a rotary microtome and affixed to glass slides. Prior to hybridization, sections were de-waxed in xylene, and re-hydrated in an ethanol series (100, 95, 85, 70, 50, 30, 15, 0% ethanol in distilled RNAse free water).

Localization of RNA was performed with ^{35}S-labelled RNA probes generated from *Ep* cDNA clones. The prehybridization, hybridization, and wash conditions followed published methods (Cox K.H., and Goldberg R.B. 1988, Analysis of plant gene expression. In Shaw CH (ed), Plant Molecular Biology: A Practical Approach, pp. 1-35. IRL Press, Oxford). Briefly, sections were treated with Proteinase K and acetylated with acetic anhydride in triethanolamine. The sections were then hybridized with ^{35}S-RNA probes overnight at 42 °C, washed, and dehydrated in an ethanol series before application of Kodak NTB-2

track emulsion. After 1 week at 4 °C, slides were developed in Kodak D-19 developer, fixed in Kodak Fix, and stained in Toluidine Blue O (0.05% in 50 mM Acetate buffer, pH 4.5). Slides were then dehydrated in an ethanol and xylene series, and mounted in Permount. Slides were photographed on Kodak EPL 400 slide film, using dark field optics

The expression of a gene under the control of the Ep (peroxidase) promoter (nucleotides 1-1532 of SEQ ID NO:2, also see co-pending US patent application serial No. 08/723,414 and 08/939,905, both of which are incorporated by reference) is localized within the hourglass cells (arrow; Figure 13(d)) within the seed-coat at 18 days after anthesis. Expression of *Ep* is first detected at 6 DPA in the thin-walled parenchyma of the outer integument, adjacent to the thick-walled parenchyma, and flanking the hilum region (Figure 13 (e)). By 9 DPA a thin band of expression extends around the entire seed coat, at the junction of the thin-and thick-walled parenchyma (Figure 13 (f)). Expression shifts to the hourglass cells as they begin to develop, at 12 DPA (Figure 13 (g)).

HP

For the analysis of HP (Figures 14 (c) and (d)), tissue samples were fixed in a solution of 50 % ethanol, 5 % acetic acid, 3.7 % formaldehyde for 3 h at room temperature, dehydrated in an ethanol series (50, 60, 70, 80, 90, 95, 100 %) then infiltrated with t-butyl alcohol (TBA) in a stepwise series (25, 50, 75, and 100 % TBA in ethanol), followed by infiltration with Paraplast by gradual addition of increasing amounts of Paraplast to 100 % TBA, followed by several changes of pure melted Paraplast at 57 °C. After infiltration, samples were placed in blocks and allowed to harden. Sections of 8 to 10 μm were cut on a rotary microtome and affixed to glass slides. Prior to hybridization, sections were de-waxed in xylene, and re-hydrated in an ethanol series (100, 95, 85, 70, 50, 30, 15, 0 % ethanol in distilled RNAse free water). Sections were then treated with

Proteinase K and acetylated with acetic anhydride in triethanolamine. Sections were hybridized with ^{35}S-RNA probes overnight at 42 °C, then washed and dehydrated in an ethanol series before application of Kodak NTB-2 track emulsion. After 1 week at 4 °C, slides were developed in Kodak D-19 developer, fixed in Kodak Fix, and briefly stained in Toluidine Blue O before dehydrating in an ethanol and xylene series, then mounting in Permount. Slides were photographed on Kodak EPL 400 slide film, using dark field optics.

The expression of a gene under the control of the HP promoter (nucleotides 1-2526 of SEQ ID NO:7) is localized within the membranous endocarp (arrow, Figure 14 (b)) at 12 days after anthesis. At six days post anthesis (DPA) expression of *HPS* is limited to the membranous inner layer of the pericarp. By 12 DPA expression is very strong and the inner epidermis is showing signs of becoming detached from the rest of the pericarp and, in places, is adhering to the seed surface. Tissue sections from this stage of development also showed strong hybridization signals in the sclerenchyma, indicating that *HP* expression occurs throughout the endocarp.

SC4

To analyse the distribution of sc4 expression with respect to cell differentiation during seed coat development *in situ* hybridization was performed on seed sections from 3-24 dpa seeds. The seed sections were hybridized with radiolabelled sense and antisense SC4 RNA probes which were detected by exposure of the sections to photographic emulsion. Within the seed sections the antisense or sense RNA probes can be localized by observing the accumulation of silver grains (produced in the emulsion by the emitted β-particles) under dark-field illumination with a light microscope. Cell walls of some plant structures can be birefringent (i.e., reflect light) under dark-field illumination. Two birefringent areas can be observed in both the hilum and the funiculus of the seed sections in

Figure 21 therefore any expression or lack there-of by sc4 will be masked in these locations.

sc4 was expressed throughout the inner integument of the seed coat at 3 dpa (Figure 21). By 6 dpa the expression pattern of sc4 had changed, and was localized to the outer integument parenchyma but not to the vascular tissue embedded within this layer. sc4 expression in the outer integument was maintained until 18 dpa after which time no further expression was detected (see Table 4 below). In concurrence with northern blot analysis, the *in situ* hybridization results revealed that sc4 expression increased to a maximum between 9-12 dpa and decreased thereafter (Table 4). In addition, expression of sc4 was not observed in the embryo of seed at 3-6 dpa.

The expression of a gene under control of the SC4 promoter (nucleotides 1-5514 SEQ ID NO:9) within soybean seed coat at 3 days after anthesis is also shown in Figure 13 (a). Expression is localized within the inner integument (arrow; Figure 13 (a)). Other areas of brightness in this figure include the recurrent vascular bundles in the funiculus, and the trichomes of the pod (the bright areas are due to the birefringence of crystalline areas in the cell walls, and are also present in the negative control; data not shown).

SC20

In situ hybridization was carried out to analyse the spatial gene expression pattern of sc20 within the seed coat between 3-24 dpa. Seed sections were hybridized with radiolabelled sense and anti-sense SC20 RNA probes. No birefringent cell structures were evident in the seed sections used (Figure 24).

Gene expression of sc20 was localized to the thick-walled parenchyma of the outer integument (see Figures 13 (b) and 24). The temporal expression pattern of 9-21 dpa expression with an observed peak at 12 dpa was almost

identical to that determined by northern blot analysis (Table 4, in Examples). sc20 transcripts were not detected in the embryo between 3-6 dpa. The *in situ* hybridization results of the seed sections concur with the northern blot analysis that within the seed organ sc20 is expressed only in the seed coat organs.

Expression of gene under control of the SC20 promoter (1-2450 of SEQ ID NO:8) is seen in Figures 13 (b) and 24.

SC21

The expression of a gene under the control of SC21 (see Figure 11 (b)) within seed coat tissues at 15 days after anthesis is localized in the thin-walled parenchyma of the outer integument, including the area immediately surrounding the tracheid bar (arrow; Figure 13 (c)).

c) Construction of genomic libraries

In order to characterise the gene corresponding to seed coat cDNA clone(s), several genomic libraries were constructed in λ vectors from total DNA isolated from etiolated seedlings of various soybean cultivars. Two soybean genomic libraries were constructed in lLambda FixII (Stratagene, La Jolla, CA) from the total DNA isolated from etiolated seedlings of soybean [Glycine max (L.) Merrill] cvs. Harosoy 63 and Harovinton. The DNA was partially digested with Bgl II prior to ligation into the cloning vector.

Genomic clones corresponding to the cDNA clone SC4 and SC20 were obtained. Lambda DNA was isolated from each plaque. An ~8 kb Xba I fragment from the SC20 lambda clone and an ~8 kb Sac I fragment from the SC4 lambda clone, identified by southern blotting, were ligated into pBlueScript-SK (Stratagene, La Jolla, CA) and transformed into E. coli TOP 10 cells.

Southern blot analysis of genomic soybean DNA, was carried out with 7 seed coat cDNA probes to determine similarities between clones and whether the clones represent a single gene or a gene family. Southerns were also performed to determine the occurrence of the seed-coat specific genes within other dicotyledonous and monocotyledonous plant species. Soybean genomic DNA was cleaved with several restriction enzymes and the resulting DNA fragments were size fractionated using agarose gel electrophoresis, denatured and transferred to nylon filters. Hybridization was carried out with radiolabelled cDNA probes.

Isolation of genomic clones

Initially, soybean genomic libraries were screened for the presence of the seed coat clone using the polymerase chain reaction with primers specifically designed from each cDNA sequence. This helped to target potential libraries for the isolation of genomic clones. The chosen genomic library was then screened using nucleic acid hybridization with cDNA probes. For genomic library screening hybridization conditions involved using modified Church's buffer (Church and Gilbert (1994) PNAS USA 81: 1991-1995) at 65°C; wash 0.1X SSC 0.1% SDS at 52-55°C. Probes were random primed in presence of ^{32}PdCTP using standard protocols.

Ep

A seed-coat peroxidase gene, corresponding to the *Ep* locus, was obtained from a soybean seed-coat library. The genomic DNA sequence comprises four exons spanning bp 1533-1752 (exon I), 2383 -2574 (exon 2), 3605-3769 (exon 3) and 4033-4516 (exon 4) and three introns comprising 1752-2382 (intron 1), 2575-3604 (intron 2) and 3770-4516 (intron 3), of SEQ ID NO:2. Features of the upstream regulatory region of the genomic DNA include a TATA box centred on bp 1487; a cap signal 32 bp down stream centred on bp 1520. Also

noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4663 and a polyadenylation site at bp 4700. The promoter region of the genomic sequence comprises nucleotides 1-1532 of SEQ ID NO:2 (see co-pending US patent application serial No. 08/723,414 and 08/939,905, both of which are incorporated by reference).

HP

For the isolation of the genomic *HP* gene, a genomic library was constructed from DNA isolated from the soybean cultivar Harosoy 63. The DNA was purified and partially digested with *Bgl* II prior to ligation into the cloning vector lambda FixII (Stratagene). The resulting library was amplified and screened with the hydrophobic protein cDNA probe (pHPScDNA1). A positive clone was identified, purified, and found to contain a 14 kb insert. The entire insert was sub-cloned into pBluescript KS(+) and named pHPS1. The *HP* gene was determined by PCR analysis to lie near one end of the 14 kb *Bgl* II fragment (for restriction map see Figure 11 (c)). This region of the pHPS1 insert was sequenced by primer walking, and 3368 bp of this sequence data is disclosed here (SEQ ID NO:7). Aside from the polyadenylation site, the cDNA sequence (pHPScDNA1) exactly matches a stretch of sequence encoded on the genomic clone (pHPS1), indicating that this gene contains no introns. Additionally, a TATA box consensus signal was identified 81 bp upstream from the ATG translation start site.

SC4

A genomic clone corresponding to SC4 cDNA clone was isolated from the soybean genomic library Harosoy 63 (Bgl II digest). The genomic sc4 clone is 8310 bp in length (SEQ ID NO:9). The promoter region is found between nucleotides 1-5514 of SEQ ID NO:9. The restriction map is provided in Figure 11 (d).

SC20

A genomic clone corresponding to SC20 cDNA clone was isolated from soybean genomic library prepared from cv Harovinton (GigapackGold packaging). The genomic sc20 clone is 7235 bp in length (see Figure 23 (a), SEQ ID NO:8). Alignment of sc20 genomic and SC20:2 cDNA sequences revealed that sc20 contained eight introns of 94 bp, 101 bp, 185 bp, 80 bp, 154 bp, 112 bp, 110 bp and 93 bp respectively (Figure 23 (a)). A search (www.hgc.lbl.gov/cgi-bin/promoter.pl) of the 5' upstream region of sc20 revealed three potential transcription start sites at positions 1085, 1156 and 2272. The promoter region is found between nucleotides 1-2450 of SEQ ID NO:8. The restriction map of SC20 is presented in Figure 11 (a) and 23(a).

SC21

A genomic clone corresponding to SC21 cDNA clone was isolated from the soybean genomic library prepared from Harosoy 63 (*EcoR*1 digest). The DNA of the SC21 genomic clone was digested with several restriction enzymes, fractionated by agarose gel electrophoresis and transferred onto nylon membrane. Hybridizations were carried out using radiolabelled cDNA. A restriction map of this clone is presented in Figure 11 (b).

Southern analysis

SC4

Southern blot analysis was carried out to examine the gene family composition of sc4. Soybean genomic DNA was cleaved with Eco RI, Hind III and Xba I. which do not have recognition sites in the SC4c cDNA sequence. Under conditions of low to high stringency (i.e., from 40-10°C below Tm of the

probe) the SC4 cDNA probe hybridized to a single band (Figure 22) and therefore sc4 appears to be a single gene.

SC20

Southern blot analysis was performed to ascertain whether sc20 is a single gene or a member of a gene family. Soybean genomic DNA was cleaved with Eco RI, Hind III, Xba I and Eco RV which have three, four, two and one recognition site(s) respectively in the sc20 clone (see Figure 23 (a)). Hybridization was carried out with radiolabelled SC20 cDNA probe which could anneal from the middle of exon 6 to the Eco RI site on exon 9. For each digest the probe was expected bind to only one of the resulting sc20 restriction fragments. Under conditions of high stringency to detect genes with at least 90% similarity to sc20 the probe hybridized to a single band (Figure. 25 (b)). Under medium stringency conditions to observe genes with 80% similarity to sc20 it was observed that the SC20 probe annealed to 2-3 bands for each digest (Figure 25 (a)). Under conditions of low stringency i.e., 40°C below Tm the SC20 probe hybridized to several more bands from each digest (data not shown). This suggested that sc20 is a member of a small gene family composed of 2-3 members and that the soybean genome contains several genes which are more distantly related to sc20.

Southern blot analysis was performed to determine the occurrence of the seed-coat genes within the following plant species: pea (*Pisum sativum*), canola (*Brassica napus*), oat (*Avena sativa*), onion (*Allium cepa*), pepper (*Capsicum annuum*), mimosa (*Mimosa pudica*), black spruce (*Picea mariana* (Mill B.S.P.), birch (*Betula pendula* Roth). Genomic DNA was cleaved with *EcoR*I and the resulting DNA fragments were fractionated using agarose gel electrophoresis, denatured and transferred to nylon filters. Hybridization was carried out with radiolabelled SC4 (Figure 22 (b)), SC20 (Figure 25 (c)), SC21, *Ep* locus peroxidase, and HP cDNA probes, using modified Church's buffer at 65°C. The

filters were washed with 2XSSC, 0.1%SDS at 42°C for 30 minutes, followed by 0.1XSSC, 0.1%SDS at 65°C for 30 minutes. SC4, SC20 and *Ep* locus peroxidase cDNA hybridized to the genomic DNA of soybean only. SC21 cDNA hybridized to the genomic DNA of both soybean and oat. HP cDNA hybridized to the genomic DNA of soybean.

Analysis of promoter activity

The developmental expression of genes under the control of SC4, SC20 SC21 and the peroxidase promoter were further characterized during development of the seed coat by *in situ* hybridization as described above. The results are summarized in Table 4.

Developmental analysis of SC20 indicates that the promoter is highly active at 12 DAF within the outer integument and thick walled parenchyma, however, activity of the SC20 promoter is detectable from about 9 DAF (as per Figure 13 (b)) to about 18 DAF, and is partially detected at 21 DAF.

The SC4 promoter is active from about 3 daf (also see Figure 13 (a)) to about 6 DAF within the inner integument, and then is highly active at 9 DAF within the outer integument and stellate parenchyma, and strongly active at 12 DAF in these same tissues. The SC4 promoter is still active within the outer integument up to 18 DAF.

The SC21 promoter is active throughout seed coat development during all stages examined, from 3 about DAF to about 24 DAF, with strongest activity noted from about 9 DAF to about 15 DAF (also see Figure 14 (c)). The gene under the control of the SC21 promoter is expressed primarily within the outer integument and derived tissues.

The Ep (peroxidase, see co-pending US patent application serial No. 08/723,414 and 08/939,905, both of which are incorporated by reference) promoter is active from about 6 DAF to about 24 DAF. Expression of the peroxidase gene, from about 12 DAF to about 24 DAF, is predominantly within cells of the outer integument, and the hourglass cells (see also Figure 13 (d)).

The HP promoter is active from about 9 daf through to about 24 daf. The promoter is active within the membranous endocarp throughout this period of time (see also Figure 14 (b)).

Table 4

Radioactive *in situ* Hybridization (^{35}S) of Soybean Seed Coat Tissue (*Glycine max* var. Maple Presto):

Developmental study with seed coat specific clones and peroxidase clones

	SC 20	SC 4	SC 21	Ep	HP
3 daf	-	+ + (inner integument)	+ (outer integument, subhilum region)	-	-
6 daf	-	+ + (outer integument)	+ (thin-walled outer integument except vascular layer; gradient from hilum to bottom of seed)	+ (localized beneath recurrent vascular bundles)	-
9 daf	+ (outer integument, thick walled parenchyma)	+ + + (outer integument except vascular layer)	+ + (thin-walled outer integument except vascular layer; gradient from hilum to bottom of seed)	+ (outer integument; thin walled parenchyma beneath vascular tissue)	+ + membranous endocarp of the pod
12 daf	+ + (outer integument, thick walled parenchyma)	+ + + (outer integument except vascular layer)	+ + (thin-walled outer integument except vascular layer)	+ + (outer integument except vascular layer; hourglass cells)	+ + + membranous endocarp of the pod
15 daf	+ (outer integument; thick & thin walled parenchyma except vascular layer)	+ (outer integument except vascular layer)	+ +(thin-walled outer integument except vascular layer)	+ + (outer integument except vascular layer; hourglass cells)	+ + + membranous endocarp of the pod
18 daf	+ (outer integument; thick & thin walled parenchyma except vascular layer)	(+)	+ + (thick & thin walled parenchyma of outer integument except vascular layer)	+ + (outer integument except vascular layer; hourglass cells)	+ + + membranous endocarp of the pod
21 daf	(+)	-	+ + (thick & thin walled parenchyma of outer integument except vascular layer)	+ + (outer integument except vascular layer; hourglass cells)	+ + + membranous endocarp of the pod
24 daf	-	-	+ + (thick & thin walled parenchyma of outer integument except vascular layer)	+ + (outer integument except vascular layer; hourglass cells)	+ + + membranous endocarp of the pod

+ + + highly expressed, many silver grains; + + moderate expression; + low expression; (+) fading expression; - no expression distinguishable

Seed Surface Analysis of Dull and Shiny Soybean Varieties

Seed surface proteins of several different soybean varieties were compared by SDS-PAGE analysis. A single seed was placed in a 2 mL plastic capped test tube and surface proteins were extracted by adding 0.5 mL of a buffer-detergent solution (10 mM Tris-Cl (pH 7.5) 0.5% SDS, 20 mM DTT) and placing the tube in a boiling water bath for 2 min. The contents of the tube were mixed and a sample was withdrawn and centrifuged for 5 min at 14,000 g. The proteins in the supernatant were electrophoretically separated on 15% acrylamide gels in the presence of SDS (Fling and Gregerson (1986) *Anal Biochem* 155, 83-88) and detected by silver staining. This analysis revealed that the 8.3 kD hydrophobic protein is by far the most abundant protein molecule occurring on the seed surface of 'Dull' seeded varieties. Only trace amounts of hydrophobic protein was detected on the surface of 'Shiny' seeded soybean varieties (results not shown).

Analysis of seed coat tissues using light microscopy indicated that the membranous endocarp of the pod wall remains in association with the seed-coat (Figure 14 (a). Scanning electron microscopy (SEM) of the seed surface of soybeans also showed obvious differences between 'Dull' (e.g.cultivar Clark) and 'Shiny' (e.g. cultivar Williams 82) varieties (see Figure 16). Whole seeds were sputter coated with gold and examined by SEM at several magnifications. When viewed with the naked eye, 'Dull' varieties present a surface with a powder-like coating whereas 'Shiny' types appear to have a smoother and more light-reflective surface. Examination by SEM at low magnification (18 X) reveals that the surface of 'Dull' types is uniformly covered with small, dimple-like indentations and bits of adhering material. These indentations are also visible on 'Shiny' types, but the surface is virtually free of adhering material. At higher SEM magnifications, the surface of 'Dull' types appears rough and ragged whereas the 'Shiny' seeded soybeans have a relatively smooth and undulating surface.

Without wishing to be bound by theory, it appears that the adhering material on the 'Dull' seeded types are remnants of the membranous endocarp tissue and is rich in hydrophobic protein. The expression of the hydrophobic protein in the endocarp causes bits of this tissue to stick to the seed surface, resulting in the 'Dull' phenotype. Lack of expression similarly may result in the 'Shiny' phenotype. The hydrophobic protein may be involved in the adherence of the endocarp to the seed surface.

Analysis of 'Dull' and 'Shiny' Seeded Varieties

Total genomic DNA was extracted from 'Dull' or 'Shiny' seeded varieties and amplified by PCR using primers targeted to the *HP* gene. The resulting amplification products were clearly polymorphic between the two genotypes. Good amplification of target segments of DNA were regularly observed when template DNA was from 'Dull' types whereas DNA from 'Shiny' types produced multiple products or products that were shorter or longer than expected, depending on the primer combination. These results indicate that different alleles the *HP* gene occurs in 'Dull' and 'Shiny' types of soybean. This allelic variation causes profound differences in seed surface morphology and composition.

To compare *HP* gene structure in two different seed luster phenotypes that were also different in the amount of HP present on the seed surfaces, we hybridized genomic DNA blots with probes derived from the HP cDNA sequence under high stringency conditions.

Soybean genomic DNA was isolated from frozen, lyophilized tissue according to the method of Dellaporta et al., (1983). Restriction enzyme digestion of 30 μg DNA, separation on 0.5 % agarose gels and blotting to nylon membranes followed standard protocols (Sambrook et al., 1989). Digoxigenin labelled cDNA was prepared and used to probe DNA blots according to the instructions provided by the manufacturer (Boehringer Mannheim).

Hybridization was carried out at 65 °C for 16 h in 0.25 M Na_2HPO_4 (pH 7.2), 20% SDS, 1 mM EDTA and 0.5% blocking reagent (Boehringer Mannheim). Filters were then washed 4 x 15 min at 22 °C in high stringency wash solution (20 mM Na_2HPO_4 (pH 7.2), 1% SDS and 1 mM EDTA), followed by 3 x 15 min washes in the same solution at 68 °C.

A typical result from such a Southern analysis is shown in Figure 18. Genomic DNA blots from cultivars that accumulated large amounts of HP on the seed surface produced strong hybridization signals. These intensely hybridizing fragments are not present in genomic DNA from plants that have only trace amounts of HP on the seed surface. However, several fainter signals are also present in DNA blots from both types of plants. These results indicate that sequences related to the HP cDNA are prevalent in the soybean genome, and that the *HP* gene structure is polymorphic among soybean cultivars. Soybean types that accumulate large amounts of HP on the seed surface possess additional copies of this gene.

All scientific publications and patent documents are incorporated herein by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing form the scope of the invention as described in the following claims.

WE CLAIM:

1. An isolated genomic DNA sequence, differentially expressed in seed coat tissues.

2. The genomic DNA sequence of claim 1 differentially expressed within the outer integument of the seed coat.

3. The genomic DNA sequence of claim 1 differentially expressed within the inner integument of the seed coat.

4. The genomic DNA sequence of claim 1 differentially expressed within the thick walled parenchyma of the seed coat.

5. The genomic DNA sequence of claim 1 differentially expressed within the thin walled parenchyma of the seed coat.

6. The genomic DNA sequence of claim 1 differentially expressed within the endothelium of the seed coat.

7. The genomic DNA sequence of claim 1 differentially expressed within the hourglass cells of the seed coat.

8. The genomic DNA sequence of claim 1 differentially expressed within the palisade of the seed coat.

9. The genomic DNA sequence of claim 1 differentially expressed within the stellate parenchyma of the seed coat.

10. The genomic DNA sequence of claim 1 differentially expressed within the membranous endocarp associated with the seed coat.

11. A seed-coat promoter obtained from the genomic DNA sequence of claim 1.

12. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the outer integument of the seed coat.

13. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the inner integument of the seed coat.

14. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the thick walled parenchyma of the seed coat.

15. The seed-coat promoter of claim 11 that provides for differential expression of a gene associated therewith, within the thin walled parenchyma of the seed coat

16. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the endothelium of the seed coat.

17. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the hourglass cells of the seed coat.

18. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the palisade of the seed coat.

19. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the stellate parenchyma the seed coat.

20. The seed-coat promoter of claim 11 that controls the differential expression of a gene associated therewith, within the membranous endocarp associated with the seed coat.

21. The isolated genomic DNA of claim 1 characterized by the restriction map selected from the group consisting of Figure 11 (a), (b), (c) and (d).

22. An isolated promoter differentially expressed in seed-coat tissues.

23. The promoter of claim 22 obtained from angiosperms.

24. The promoter of claim 23 obtained from the group consisting of tobacco or soybean.

25. A cloning vector comprising a heterologous gene encoding a protein, and the promoter of claim 22, wherein the heterologous gene is under the control of the promoter.

26. A plant cell which has been transformed with a vector as claimed in claim 25.

27. A transgenic plant cell containing a promoter as claimed in claim 22, operatively linked to a heterologous gene encoding a protein.

28. A seed containing a promoter as claimed in claim 22, operatively linked to a heterologous gene encoding a protein.

29. An isolated genomic DNA sequence, preferentially expressed in seed coat tissues.

30. A seed-coat promoter obtained from the genomic DNA sequence of claim 29.

31. The seed-coat promoter of claim 11 comprising at least 10 contiguous nucleotides of nucleotides 1-2526 of SEQ ID NO:7.

32. The seed coat promoter of claim 31 comprising nucleotides 1-2526 of SEQ ID NO:7, or an analogue thereof, wherein said analogue hybridizes to a nucleic acid defined by nucleotides 1-2526 of SEQ ID NO:7 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

33. The seed-coat promoter of claim 11 comprising at least 10 contiguous nucleotides of nucleotides 1-2450 of SEQ ID NO:8.

34. The seed coat promoter of claim 33 comprising nucleotides 1-2450 of SEQ ID NO:8, or an analogue thereof, wherein said analogue hybridizes to a nucleic acid defined by nucleotides 1-2450 of SEQ ID NO:8 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

35. The seed-coat promoter of claim 11 comprising at least 10 contiguous nucleotides of nucleotides 1-5514 of SEQ ID NO:9.

36. The seed coat promoter of claim 35 comprising nucleotides 1-5514 of SEQ ID NO:9 or an analogue there, wherein said analogue hybridizes to a nucleic acid defined by nucleotides 1-5514 of SEQ ID NO:9 under stringent hybridization conditions and maintains seed-coat, or seed-coat associated promoter activity.

37. A cloning vector comprising a heterologous gene encoding a protein, and the promoter of any one of claims 32, 34 or 36 wherein the heterologous gene is under the control of the promoter.

38. A plant cell which has been transformed with a vector as claimed in claim 37.

39. A transgenic plant cell containing a promoter as claimed in claim 38, operatively linked to a heterologous gene encoding a protein.

40. A seed containing a promoter as claimed in any one of claims 32, 34 or 36, operatively linked to a heterologous gene encoding a protein.

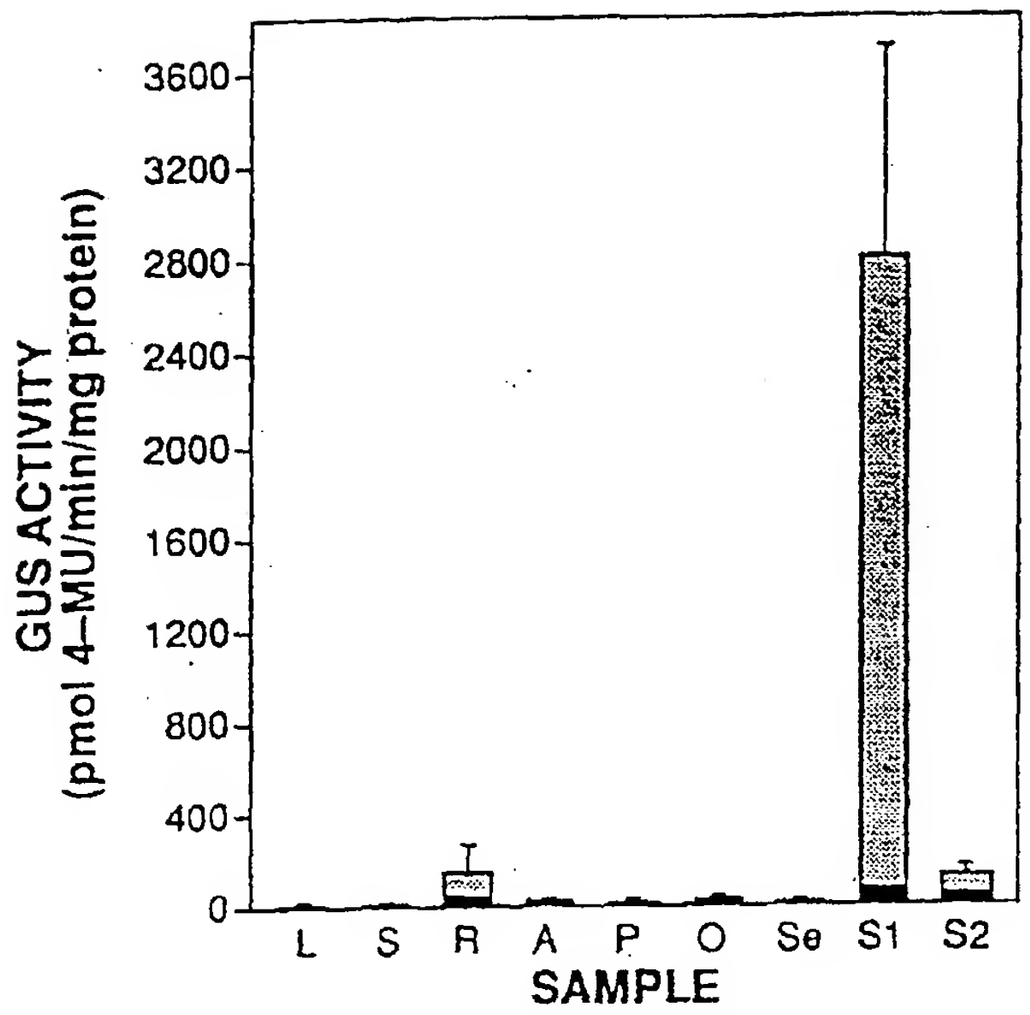

GUS ACTIVITY
(pmol 4–MU/min/mg protein)
3600
3200
2800
2400
2000
1600
1200
800
400
0
L
S
R
A
P
O
Se
S1
S2
SAMPLE

FIGURE 1

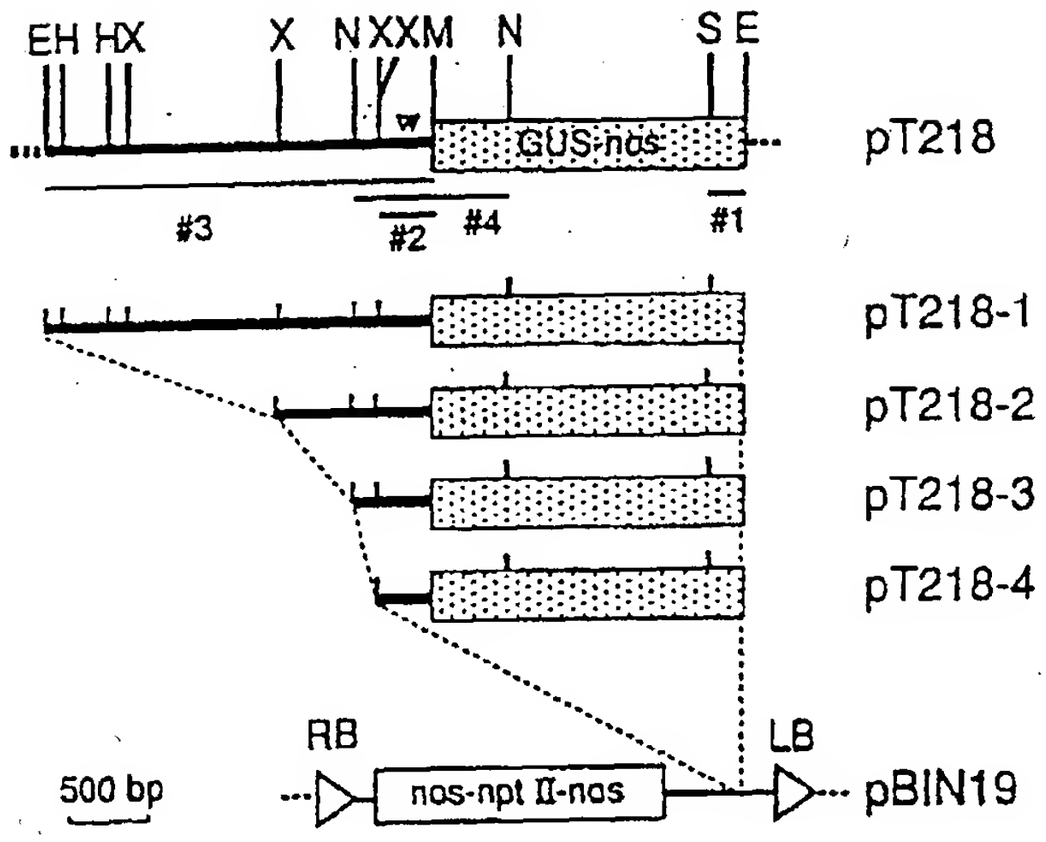


FIGURE 2

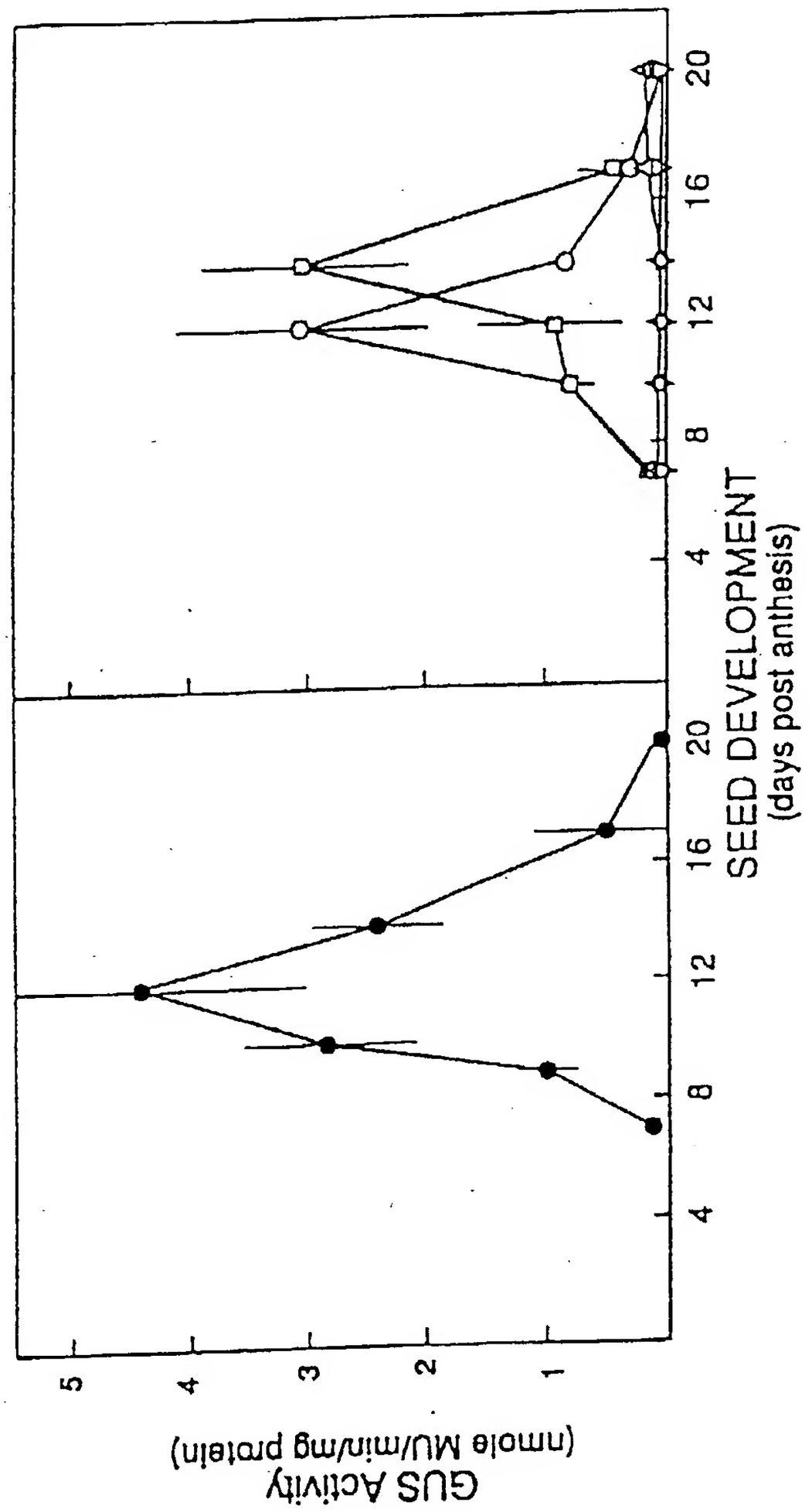


FIG. 3A

FIG. 3B

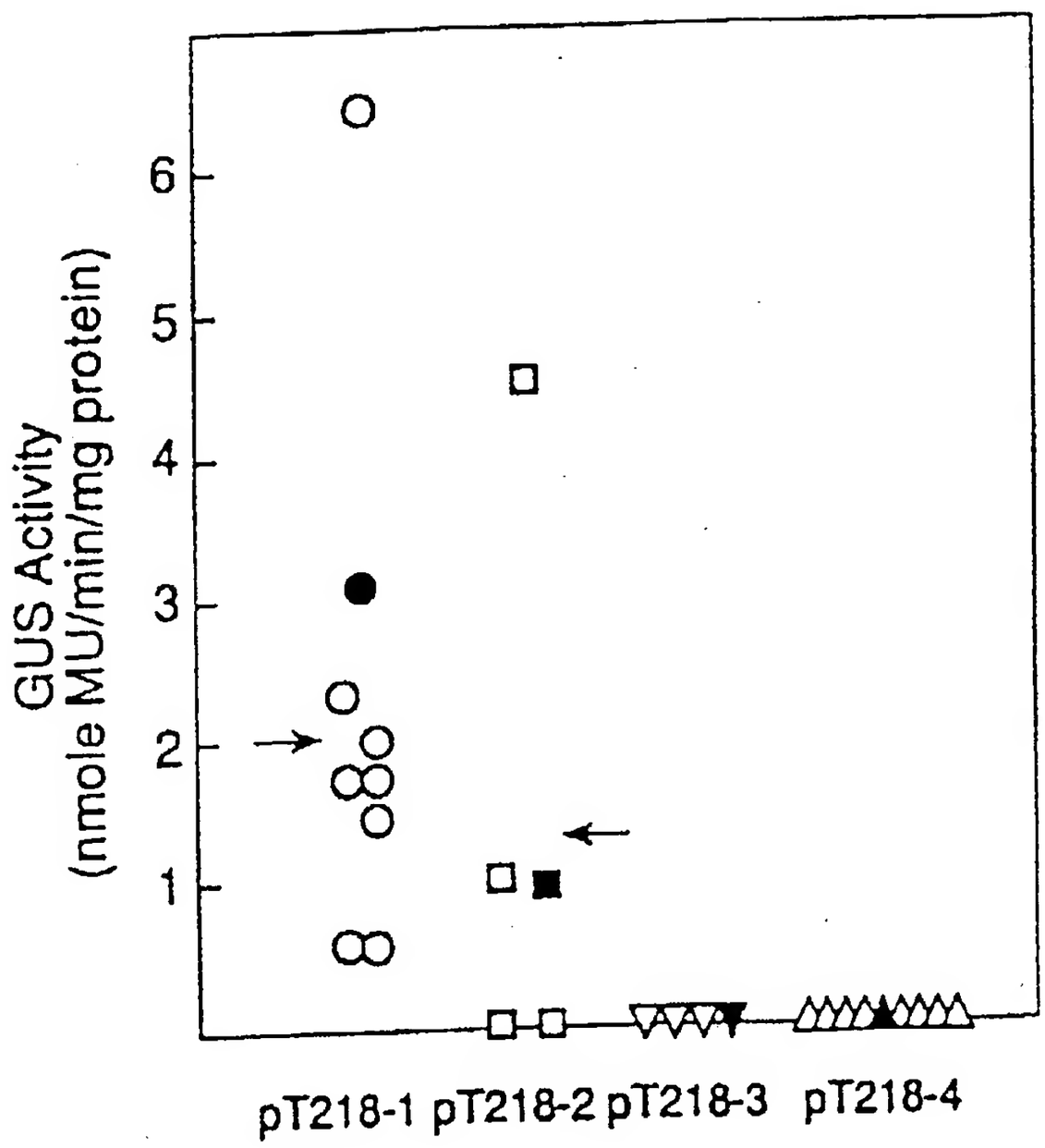


FIGURE 4

WEST

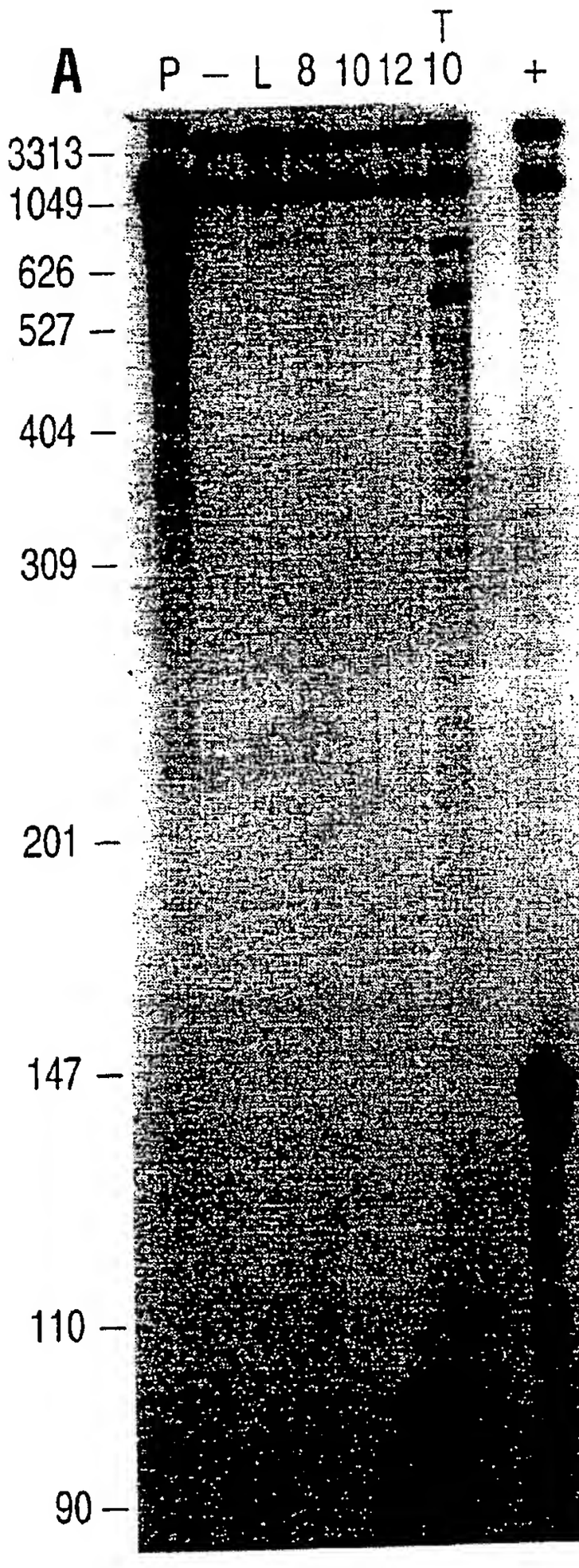


FIG. 5A

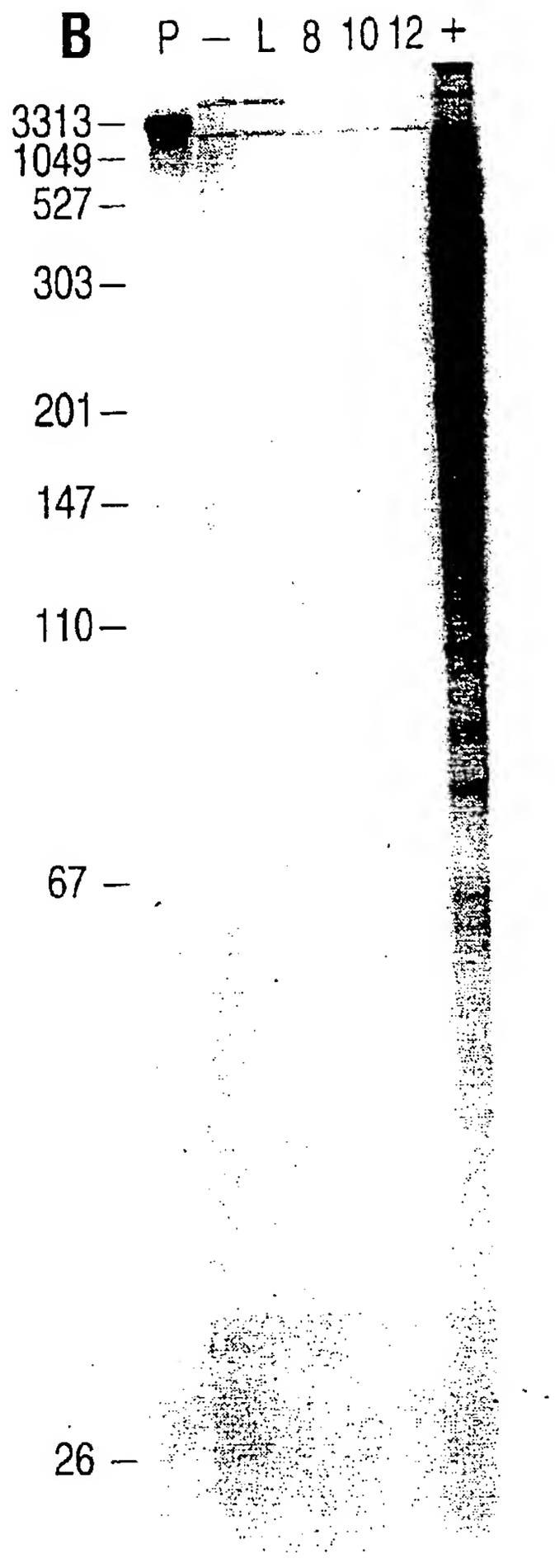


FIG. 5B

WEST

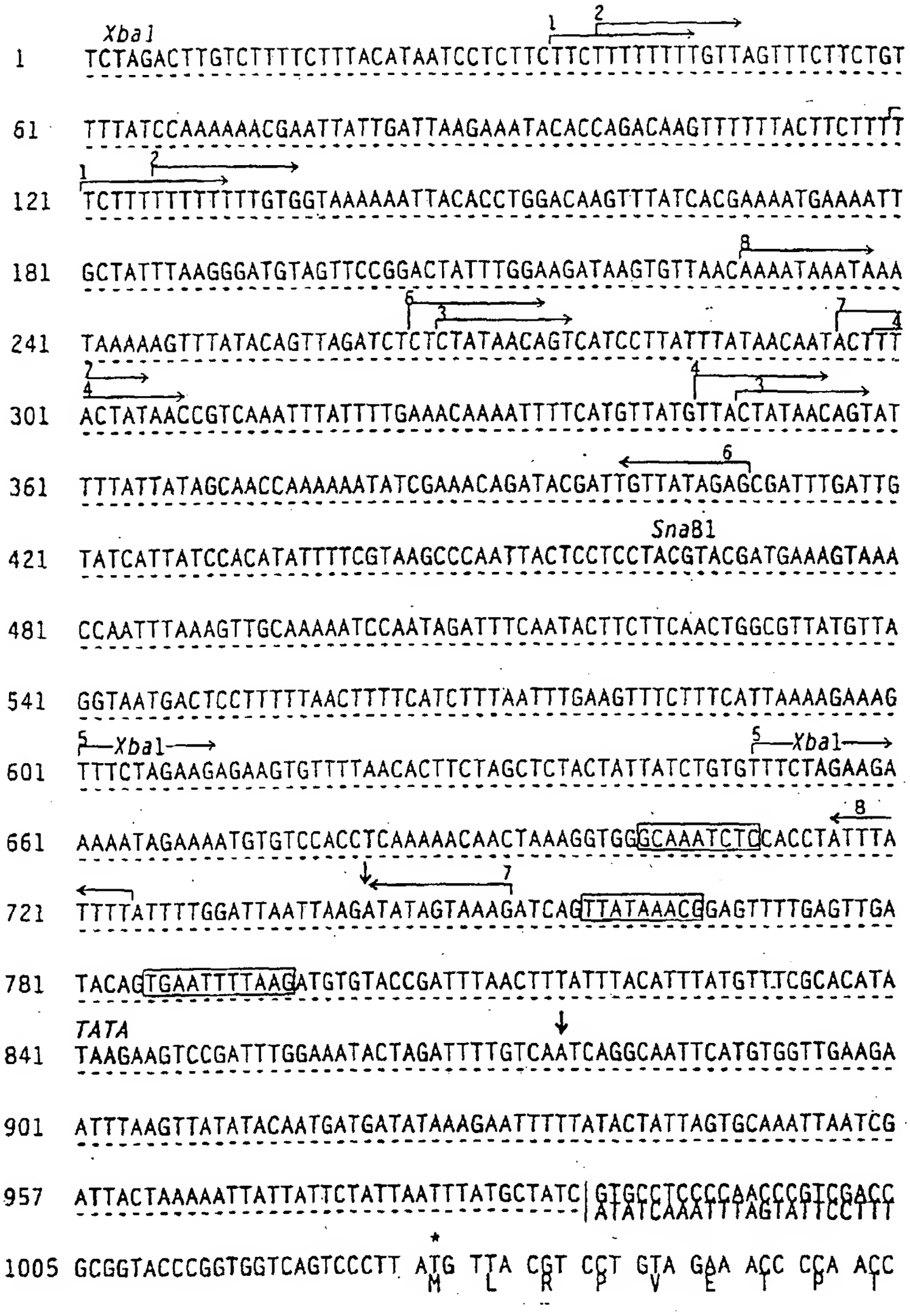

XbaI
SnaBI
XbaI
XbaI
TATA
1 TCTAGACTTGTCTTTTCTTTACATAATCCTCTTCTTCTTTTTTTTTGTTAGTTTCTTCTGT
61 TTTATCCAAAAAACGAATTATTGATTAAGAAATACACCAGACAAGTTTTTTACTTCTTTT
121 TCTTTTTTTTTTTGTGGTAAAAAATTACACCTGGACAAGTTTATCACGAAAATGAAAATT
181 GCTATTTAAGGGATGTAGTTCCGGACTATTTGGAAGATAAGTGTTAACAAAATAAATAAA
241 TAAAAAGTTTATACAGTTAGATCTCTCTATAACAGTCATCCTTATTTATAACAATACTTT
301 ACTATAACCGTCAAATTTATTTTGAAACAAAATTTTCATGTTATGTTACTATAACAGTAT
361 TTTATTATAGCAACCAAAAAATATCGAAACAGATACGATTGTTATAGAGCGATTTGATTG
421 TATCATTATCCACATATTTTCGTAAGCCCAATTACTCCTCCTACGTACGATGAAAGTAAA
481 CCAATTTAAAGTTGCAAAAATCCAATAGATTTCAATACTTCTTCAACTGGCGTTATGTTA
541 GGTAATGACTCCTTTTTAACTTTTCATCTTTAATTTGAAGTTTCTTTCATTAAAAGAAAG
601 TTTCTAGAAGAGAAGTGTTTTAACACTTCTAGCTCTACTATTATCTGTGTTTCTAGAAGA
661 AAAATAGAAAATGTGTCCACCTCAAAAACAACTAAAGGTGGGCAAATCTCCACCTATTTA
721 TTTTATTTTGGATTAATTAAGATATAGTAAAGATCAGTTATAAACGGAGTTTTGAGTTGA
781 TACAGTGAATTTTAAGATGTGTACCGATTTAACTTTATTTACATTTATGTTTCGCACATA
841 TAAGAAGTCCGATTTGGAAATACTAGATTTTGTCAATCAGGCAATTCATGTGGTTGAAGA
901 ATTTAAGTTATATACAATGATGATATAAAGAATTTTTATACTATTAGTGCAAATTAATCG
957 ATTACTAAAAATTATTATTCTATTAATTTATGCTATC|GTGCCTCCCCAACCCGTCGACC
ATATCAAATTTAGTATTCCTTT
1005 GCGGTACCCGGTGGTCAGTCCCTT ATG TTA CGT CCT GTA GAA ACC CCA ACC
M L R P V E T P T

FIGURE 6

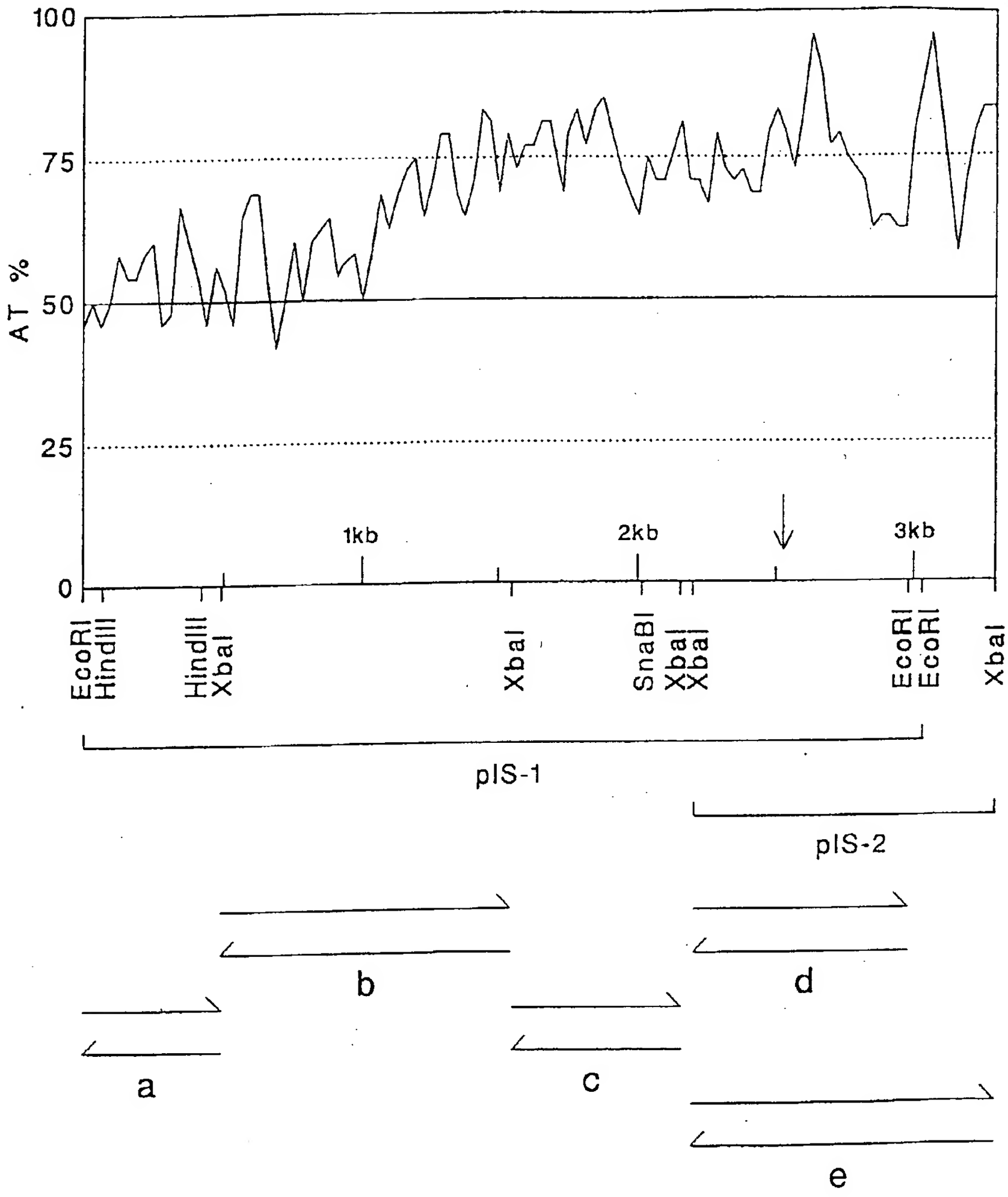


FIGURE 7

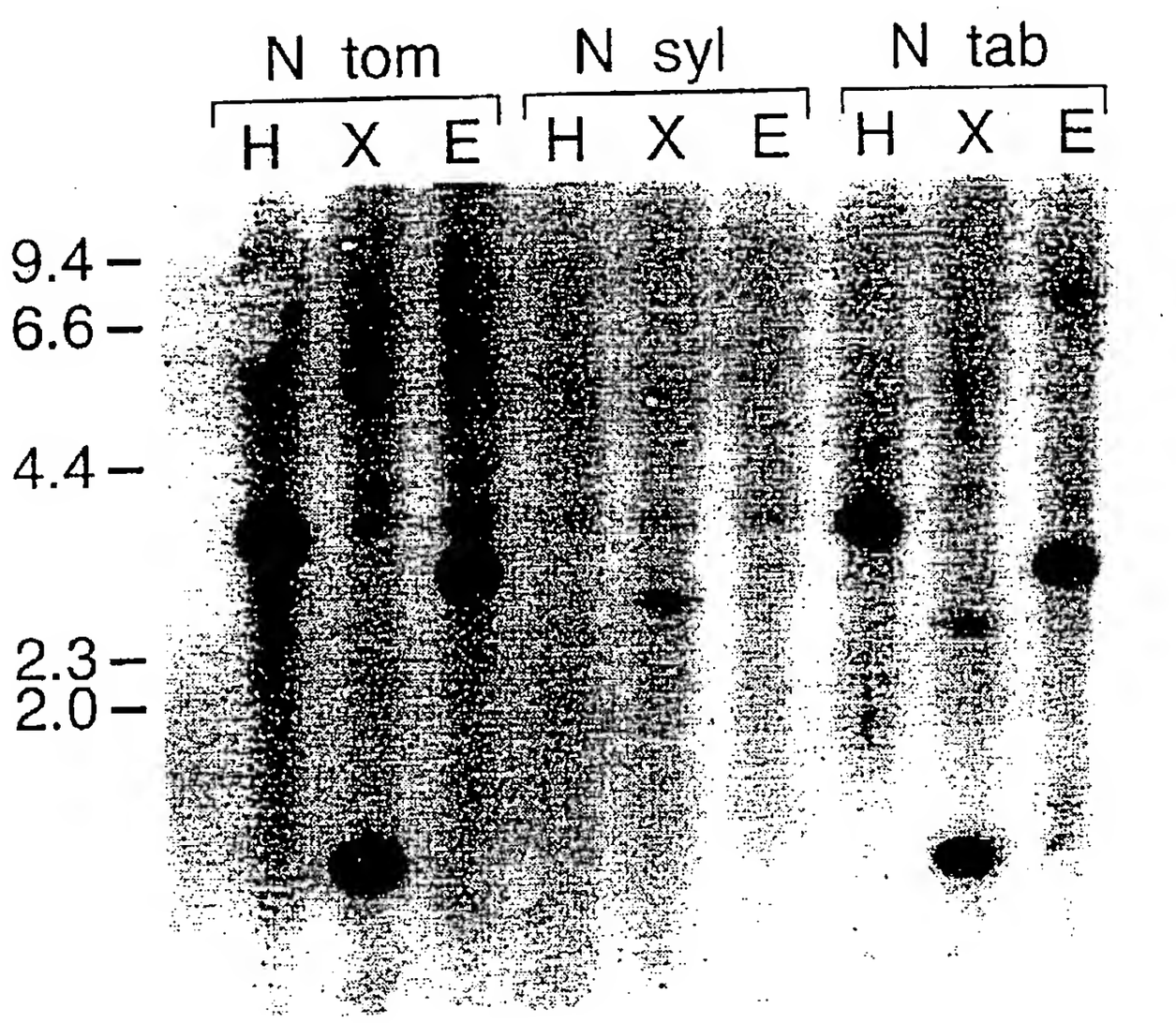


FIG. 8

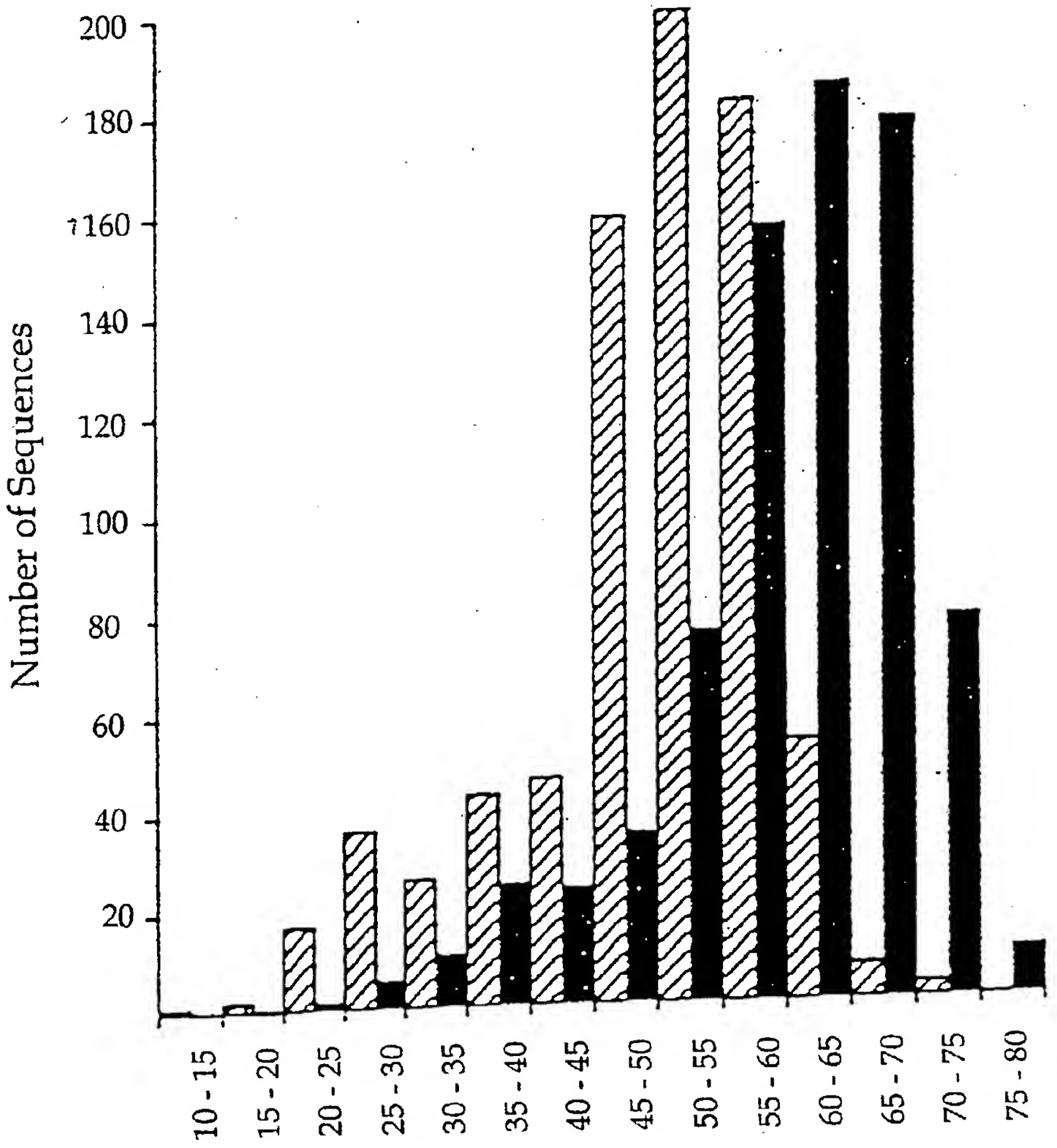
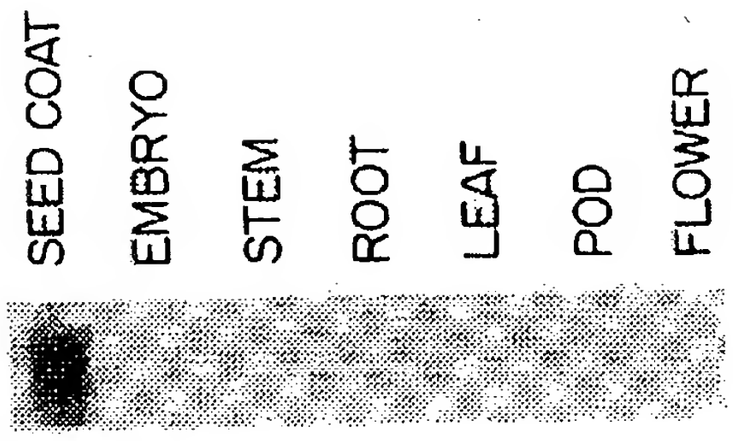
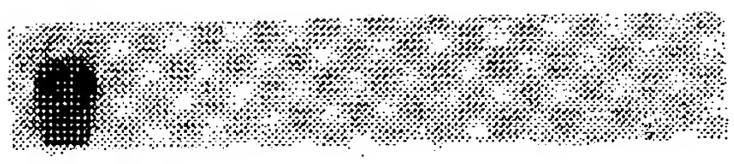


FIGURE 9



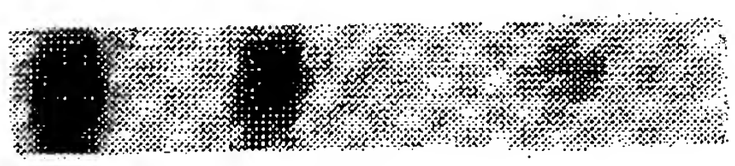
SC4

FIG. 10A



SC20

FIG. 10B



SC21

FIG. 10C

EP LOCUS
PEROXIDASE

FIG. 10D

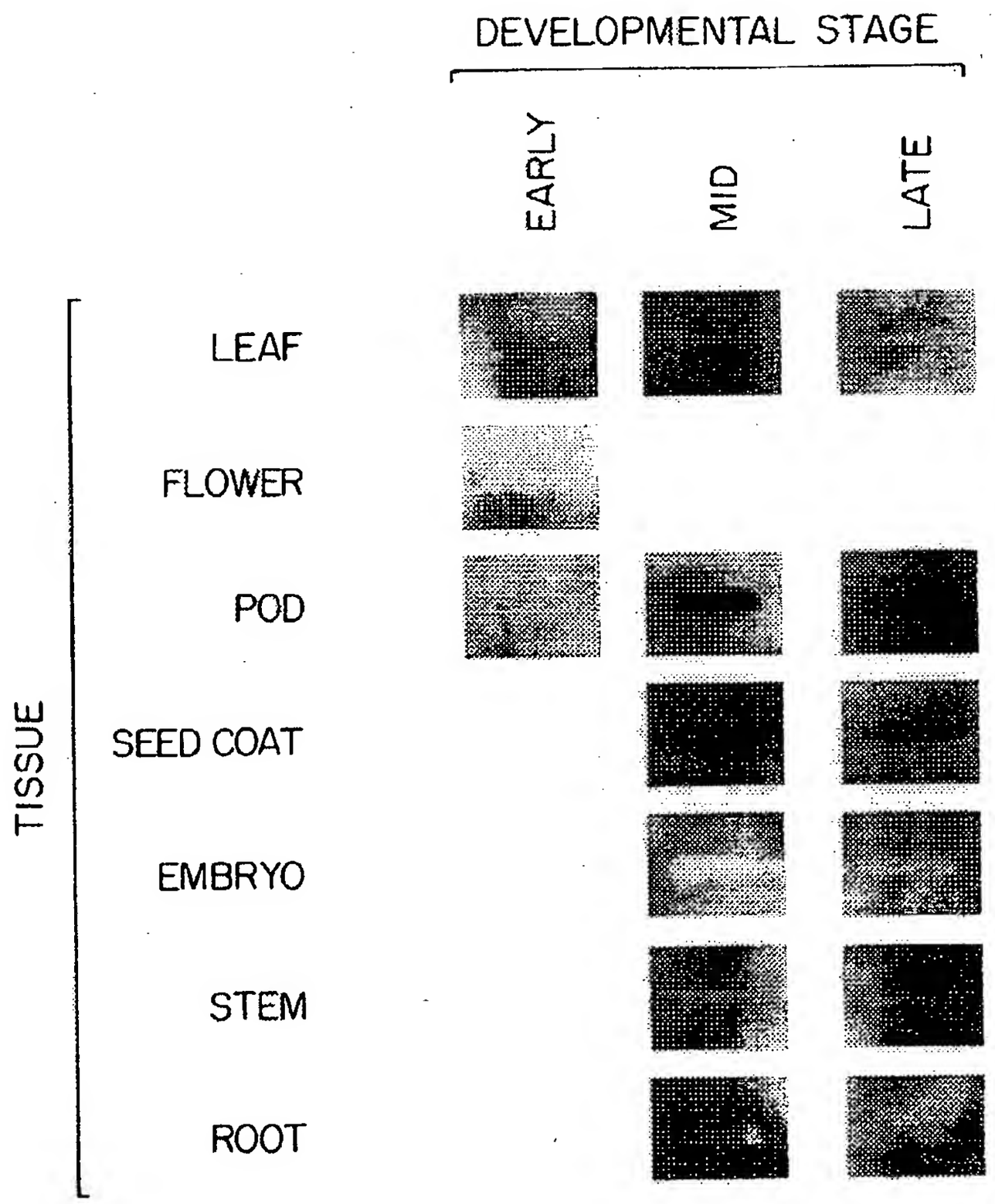

DEVELOPMENTAL STAGE
EARLY
MID
LATE
TISSUE
LEAF
FLOWER
POD
SEED COAT
EMBRYO
STEM
ROOT

FIG. 10E

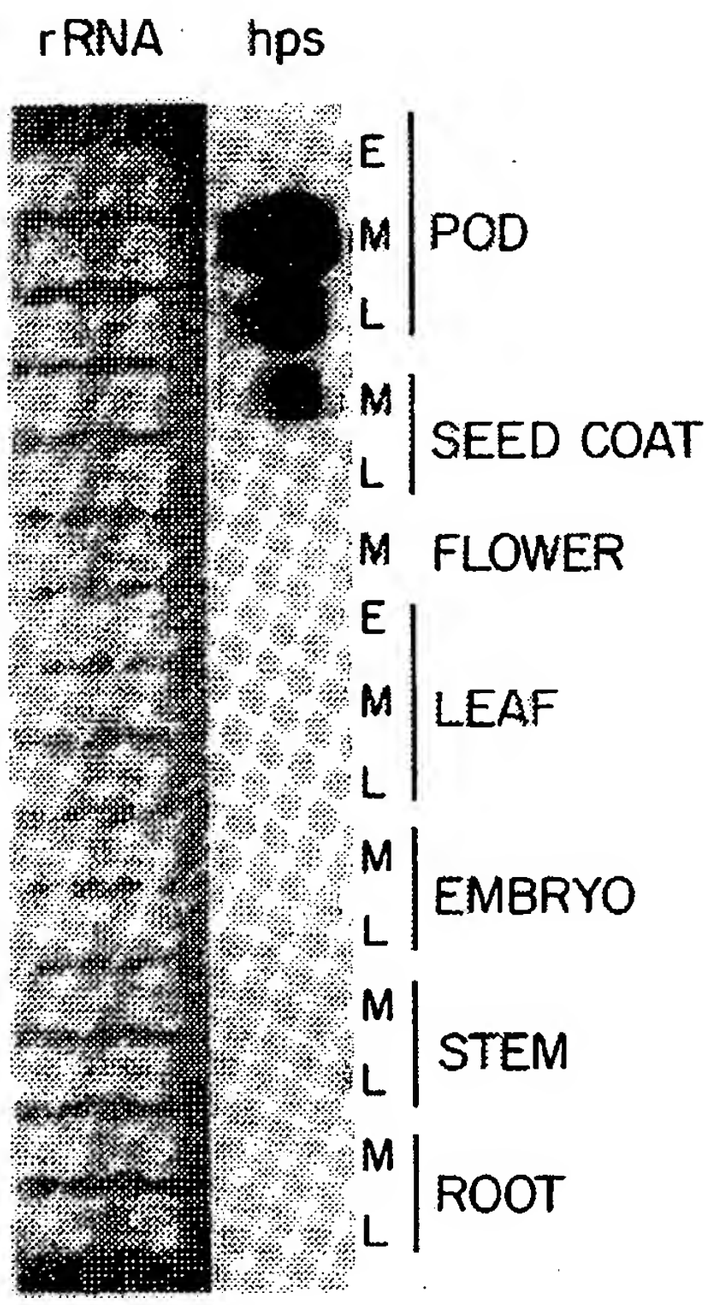


FIG. 10F

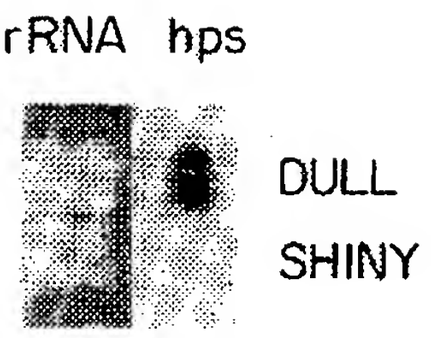


FIG. 10G

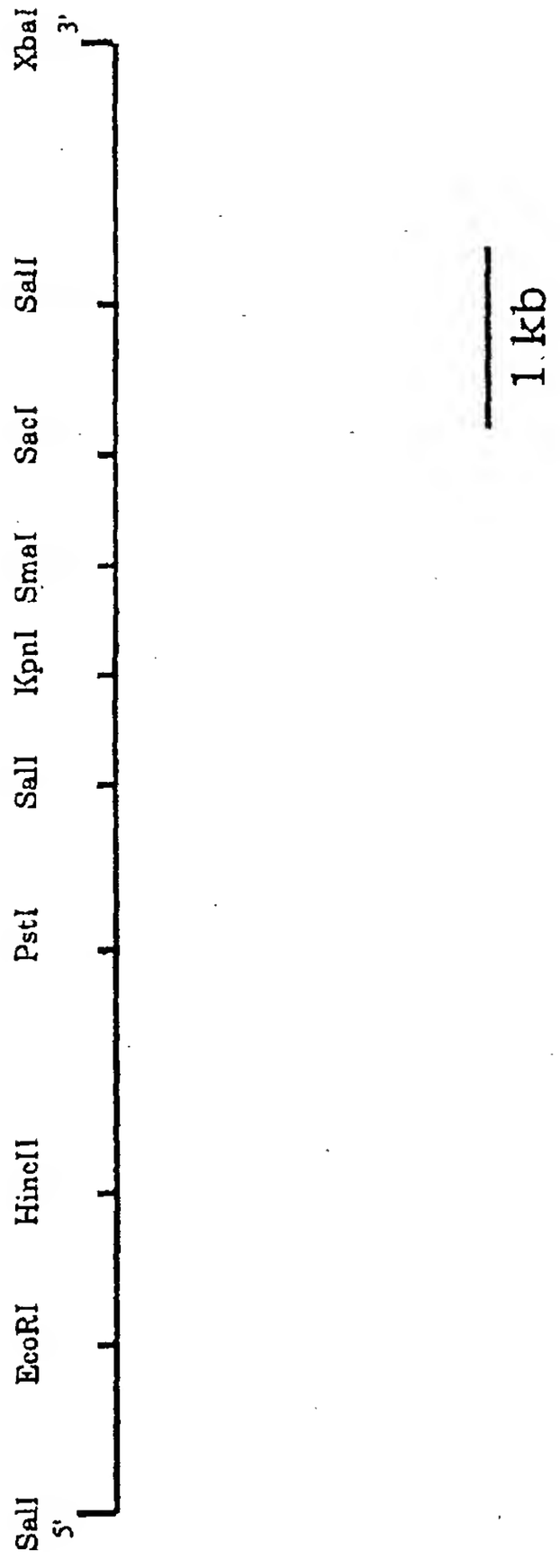


FIGURE 11(A)

WEST

RESTRICTION MAP OF SC21

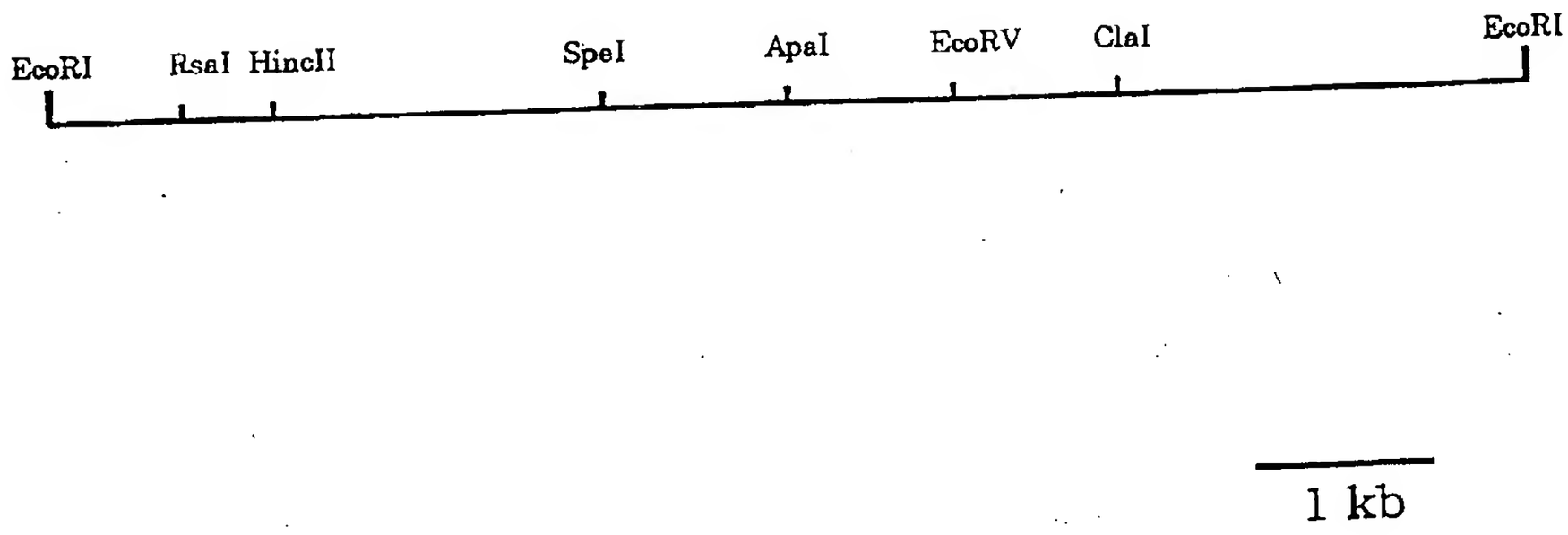


FIGURE 11(B)

WEST

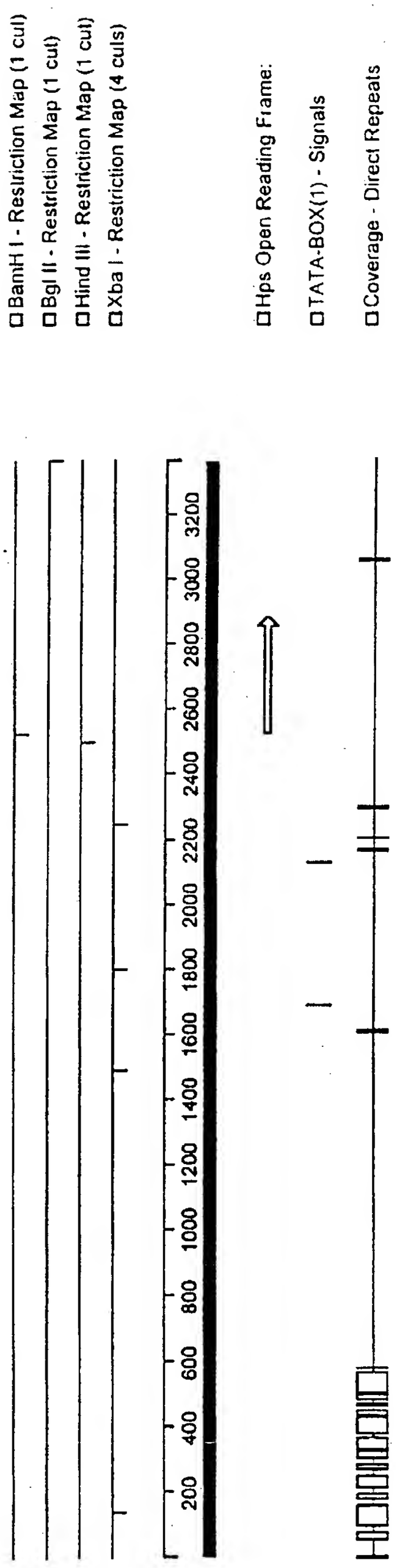
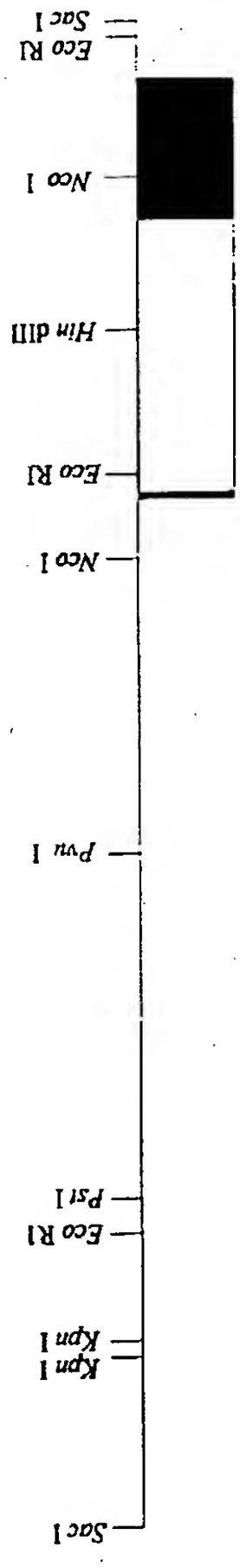


FIGURE 11(C)

WEST



Restriction map of *sc4*

The shaded and open boxes represent exons and introns respectively.

FIG. 11(D)

6 DAF

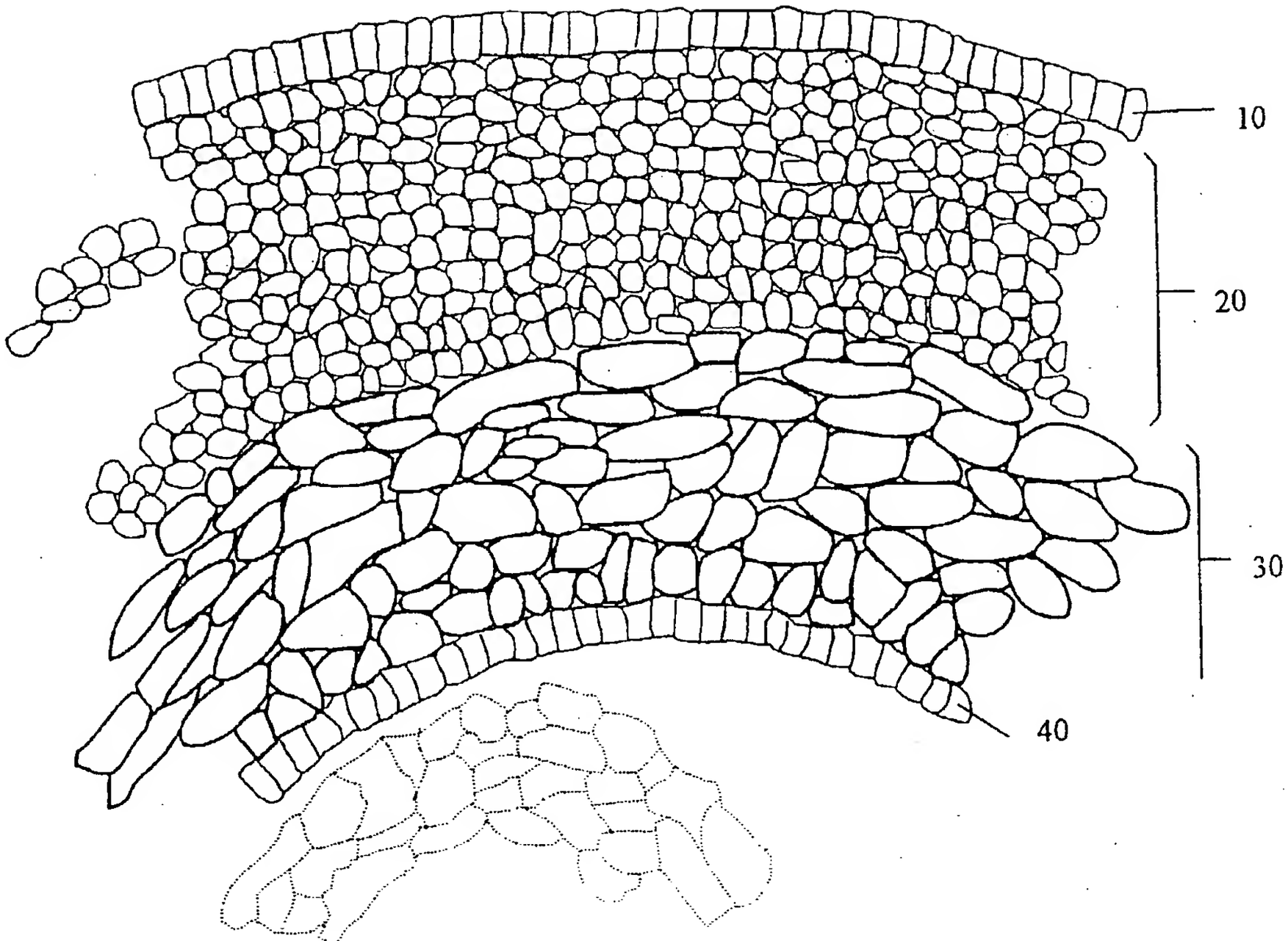


FIGURE 12(a)

WEST

12 DAF

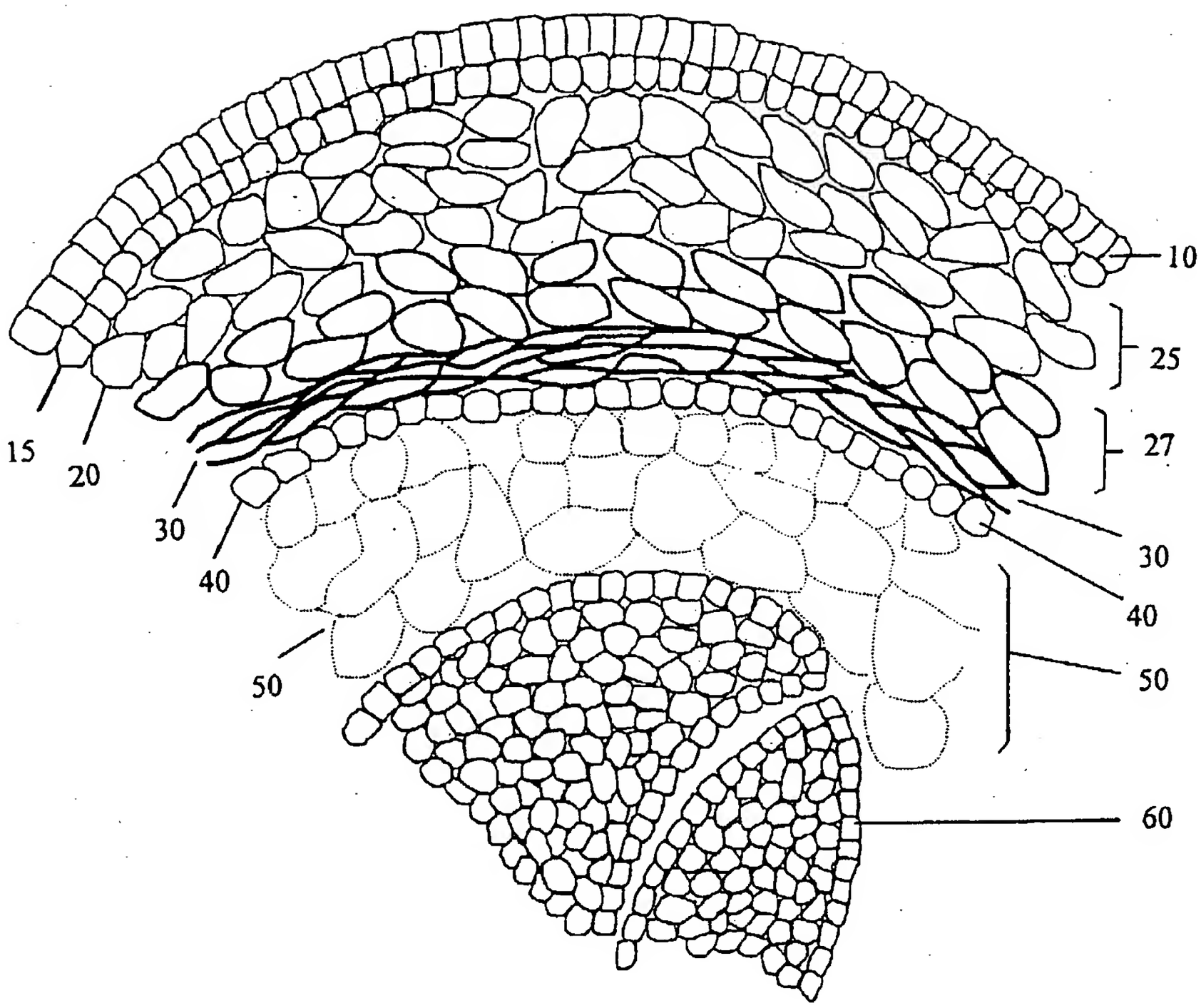


FIGURE 12(b)

WEST

18 DAF

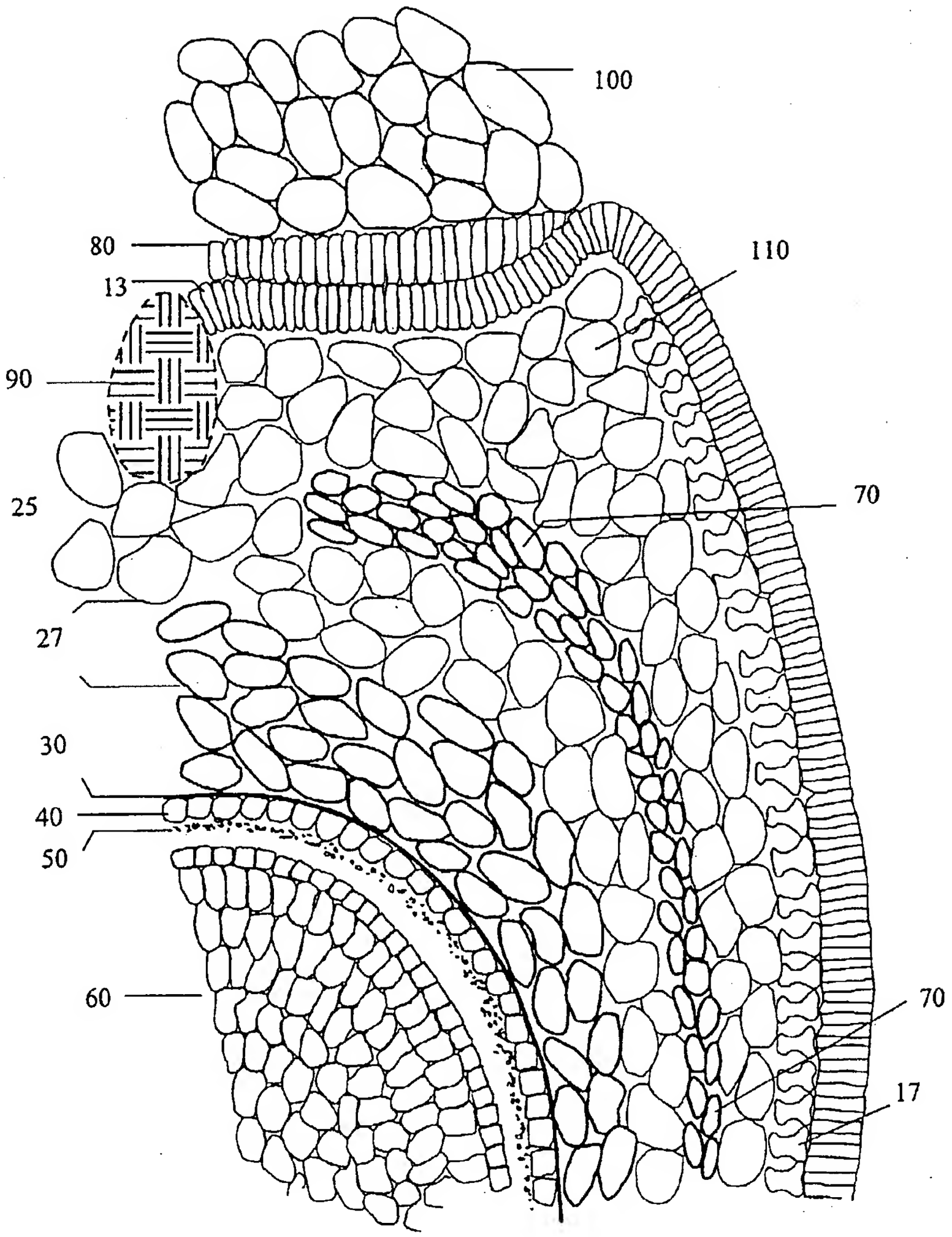


FIGURE 12(c)

WEST

FIG. 13A

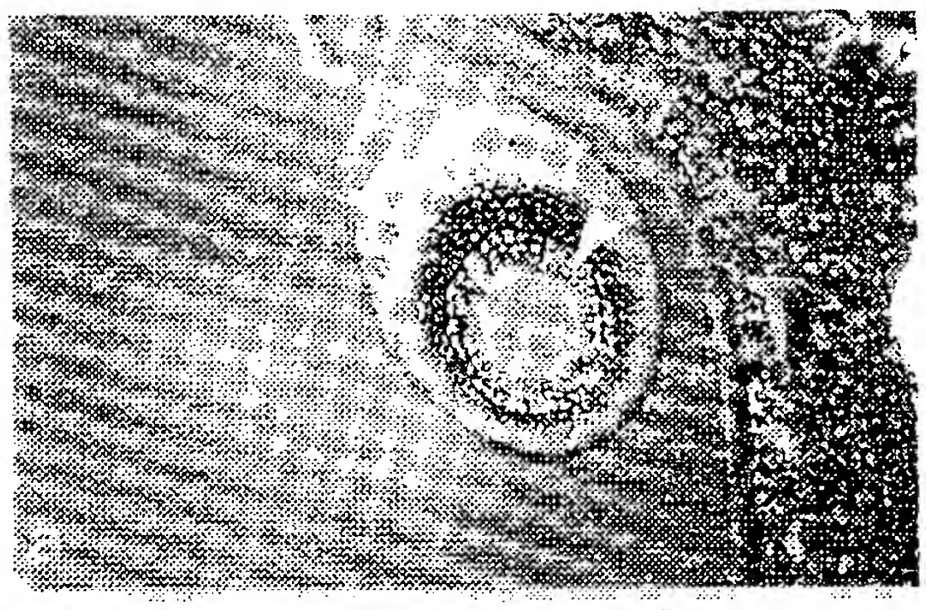


FIG. 13B

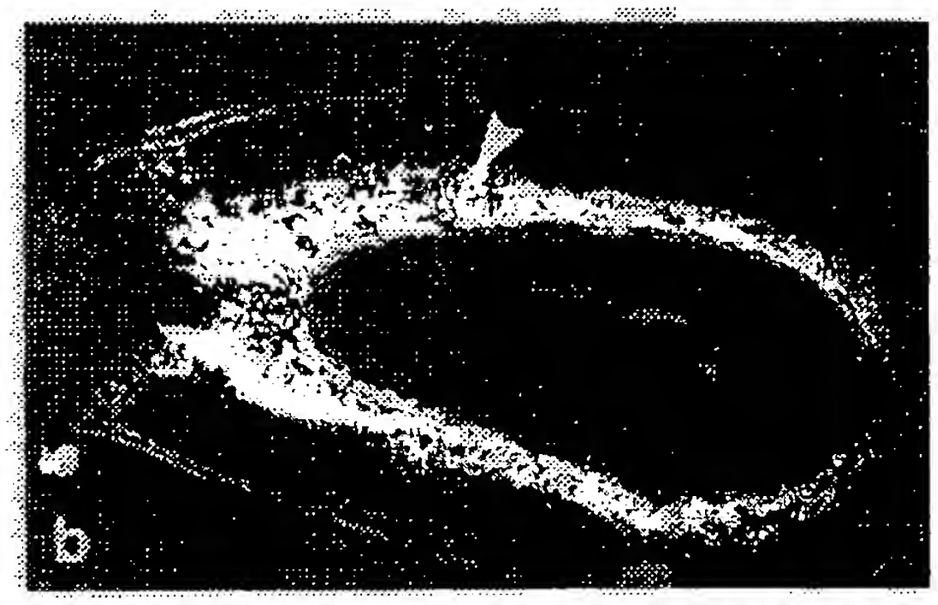


FIG. 13C

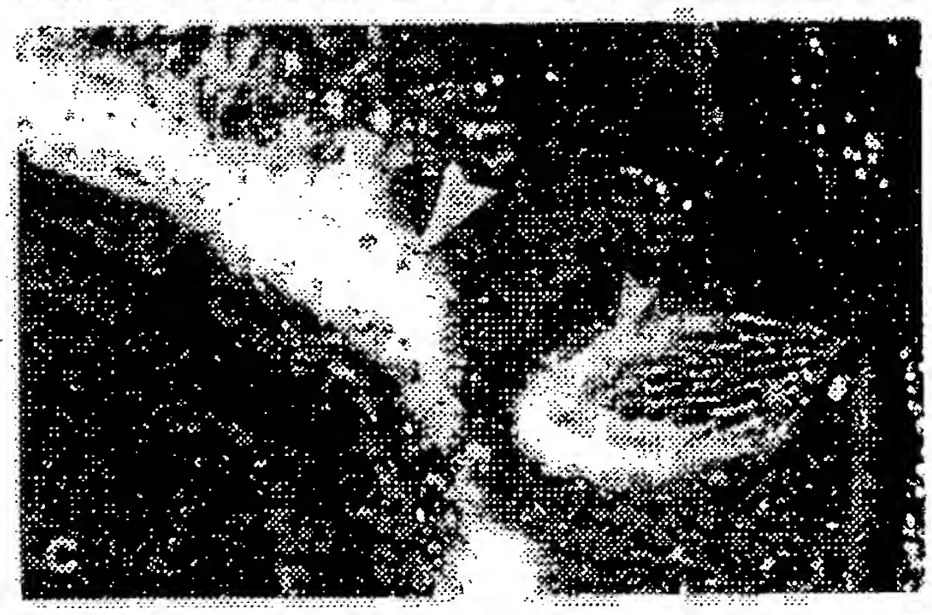


FIG. 13D

FIG. 13E

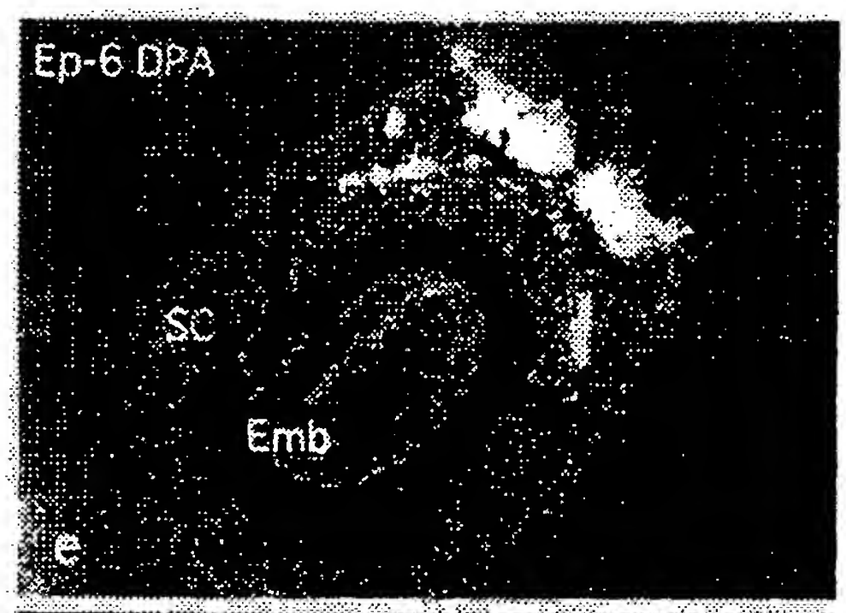


FIG. 13F

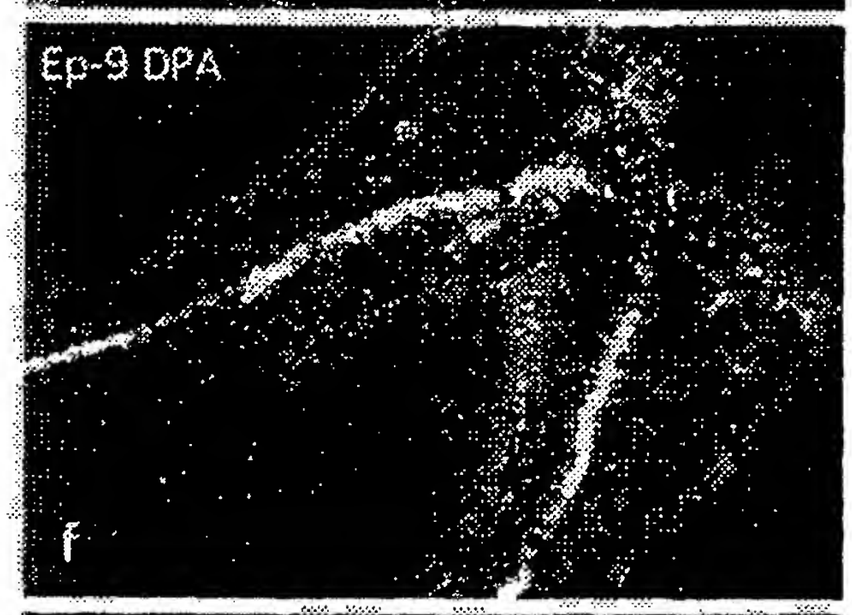
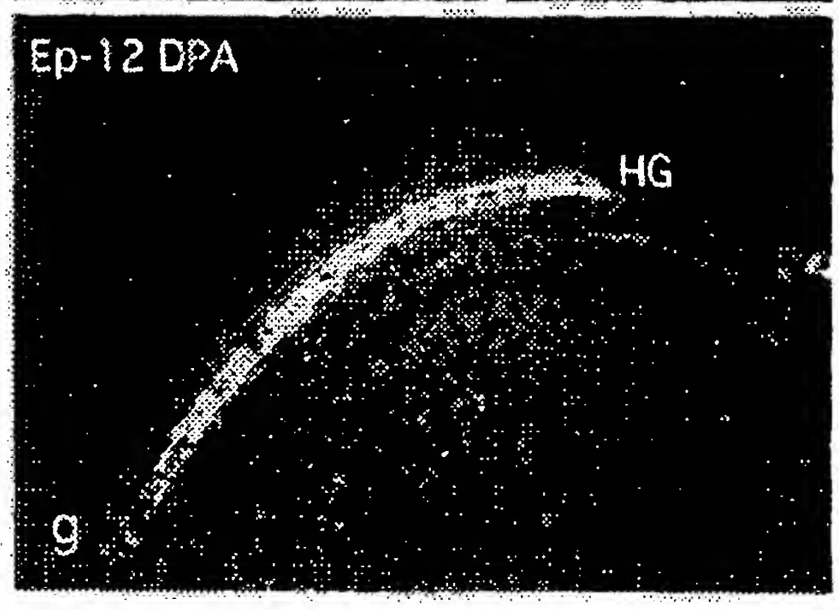


FIG. 13G



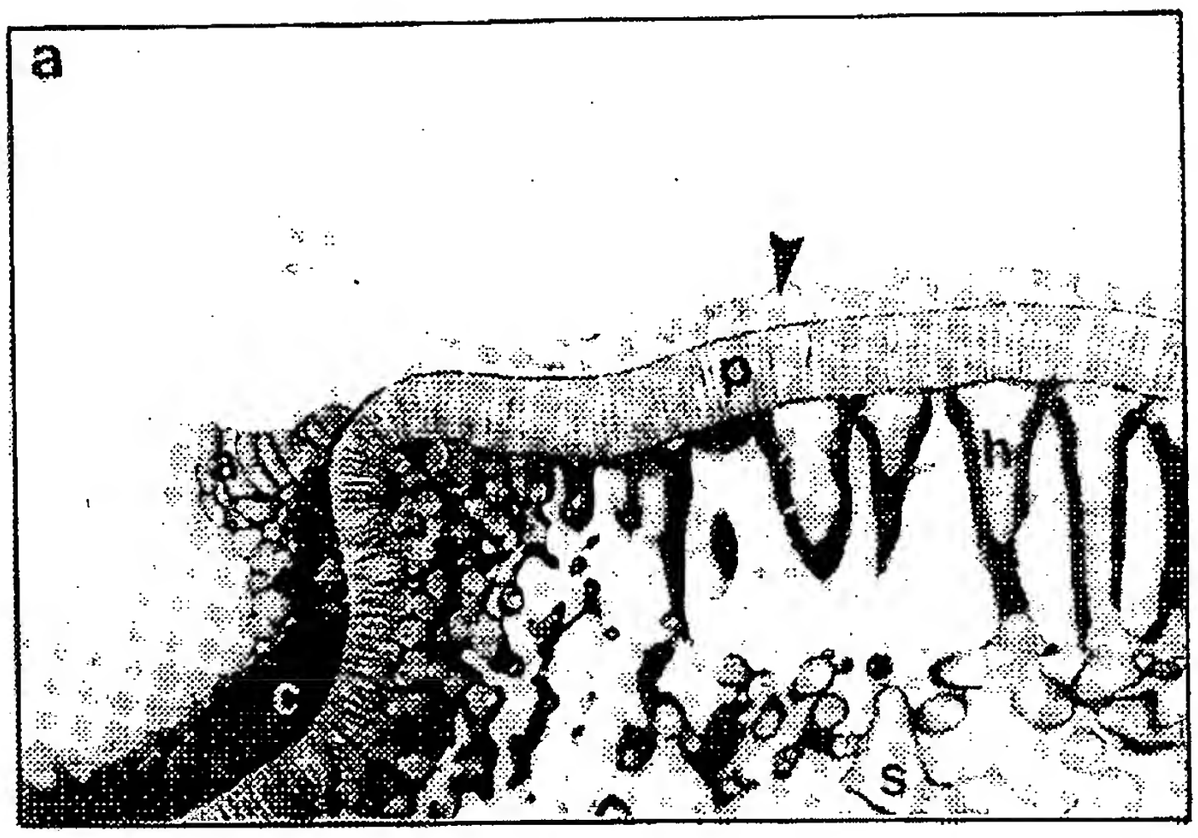


FIG. 14A

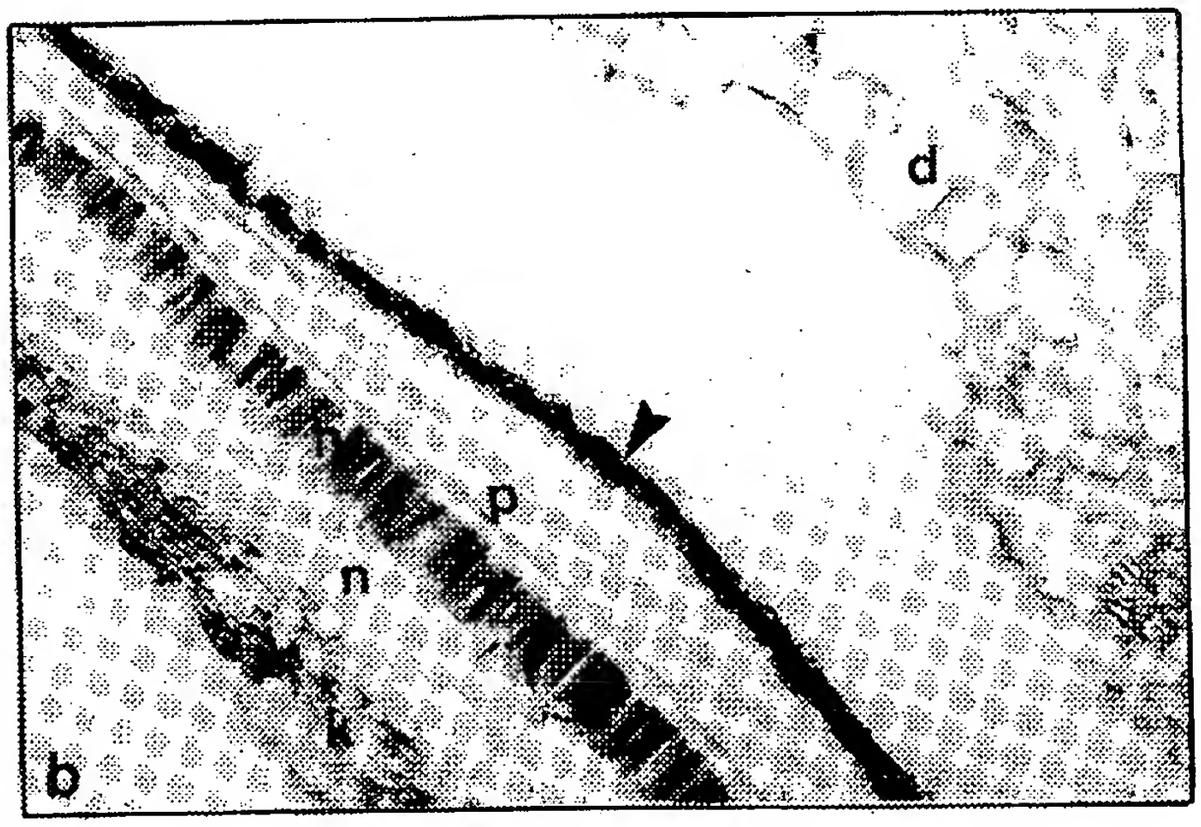


FIG. 14B

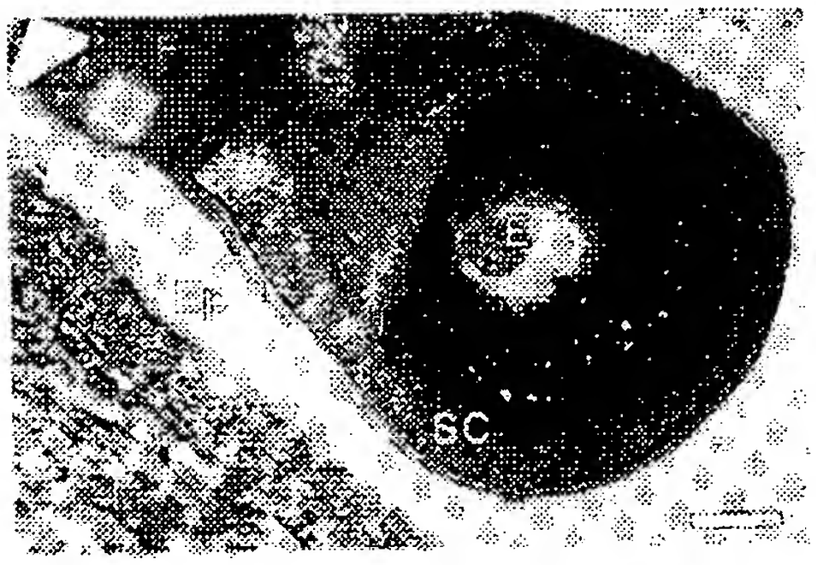


FIG. 14C

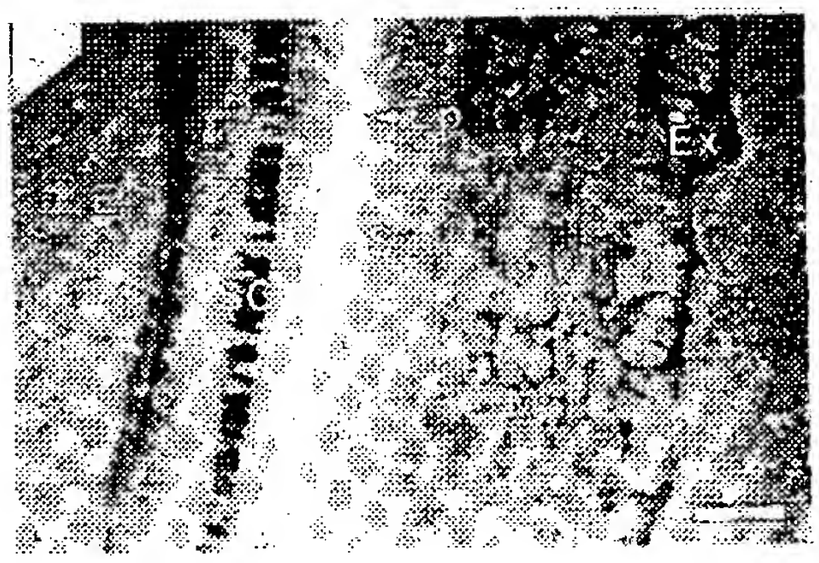


FIG. 14D

```
TAAGCTTTCAAGAGACAAACTGCTTTGAAAAATGGGATCCAAGGTTGTTGCATCCGTTGC    60
                                M  G  S  K  V  V  A  S  V  A     10

CCTTCTCCTCTCCATCAACATTCTTTTCATTTCCATGGTTAGCTCCAGCAGCCACTACGA   120
  L  L  L  S  I  N  I  L  F  I  S  M  V  S  S  S  S  H  Y  D     30

TCCACAGCCCCAACCTTCTCACGTCACTGCTCTTATTACACGACCTAGTTGTCCGGATCT   190
  P  Q  P  Q  P  S  H  V  T  A  L  I  T  R  P  S  C  P  D  L     50

GAGTATTTGCCTCAATATTTTAGGCGGGTCTCTAGGAACCGTGGATGATTGTTGTGCCCT   240
  S  I  C  L  N  I  L  G  G  S  L  G  T  V  D  D  C  C  A  L     70

CATCGGTGGTCTTGGTGACATTGAAGCCATTGTGTGCCTTTGCATCCAACTCAGGGCCCT   300
  I  G  G  L  G  D  I  E  A  I  V  C  L  C  I  Q  L  R  A  L     90

CGGAATATTAAACCTTAACCGTAATTTGCAGTTAATATTAAACTCCTGTGGACGAAGCTA   360
  G  I  L  N  L  N  R  N  L  Q  L  I  L  N  S  C  G  R  S  Y    110

CCCGTCAAACGCCACTTGCCCCCGAACCTAAGAACAGAATATGTATGGCACTAATTACCA   420
  P  S  N  A  T  C  P  R  T  *                                  119

TATTACTTCGTATCATGGTGTTTGTTTGTTTGTCTGTGTTTAAAGTTAAGGATGTTATAC   480

CCTTCGTGCCTGCTACATATATATAGTGGGCACTATAATATTACCAATAAATTAACGTCC   540

ATATATAAGAATAATAATAAATAAATAAATATTTCTATACAAATAAAGGTTACGTAATGT   600

TGTTGTTCTCGTGGATGGGGATCTTATCTTCCTCCTCGCTATCTTTGTTTATCGTATTTC   660

AGTGAAAGTTGTTCAATAAAAGTCCTTTGTTCAACAAGT(A)n                    700
```

FIGURE 15(A)

```
MGSKVVASVALLLSINILFISMVSS   25
SSHYDPQPQPSHVTALITRPSCPDL   50
SICLNILGGSLGTVDDCCALIGGLG   75
DIEAIVCLCIQLRALGILNLNRNLQ  100
LILNSCGRSYPSNATCPRT        119
```

FIG. 15(B)

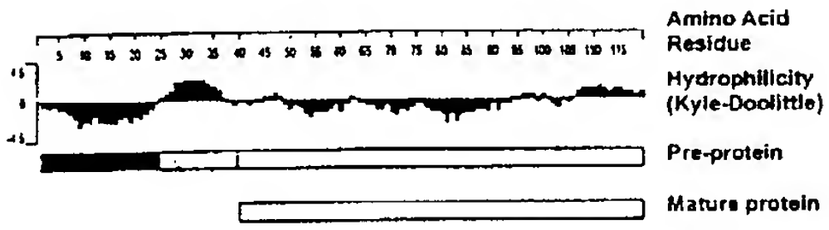


FIG. 15(C)

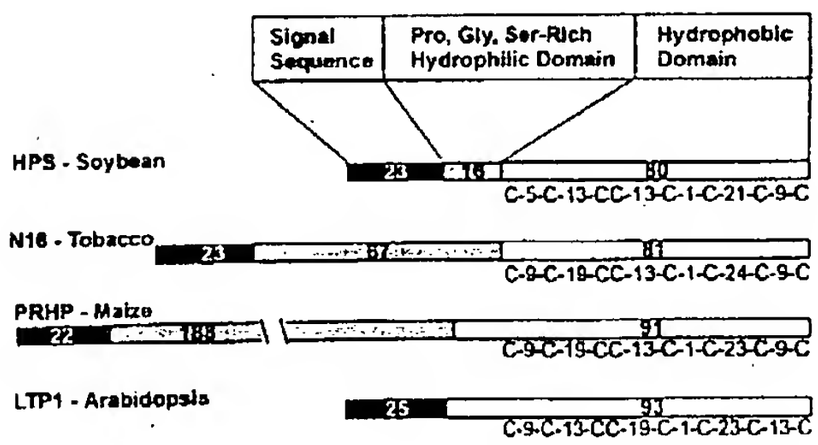


FIG. 15(D)

FIG. 15

CULTIVAR: CLARK
PHENOTYPE: DULL

WILLIAMS 82
SHINY

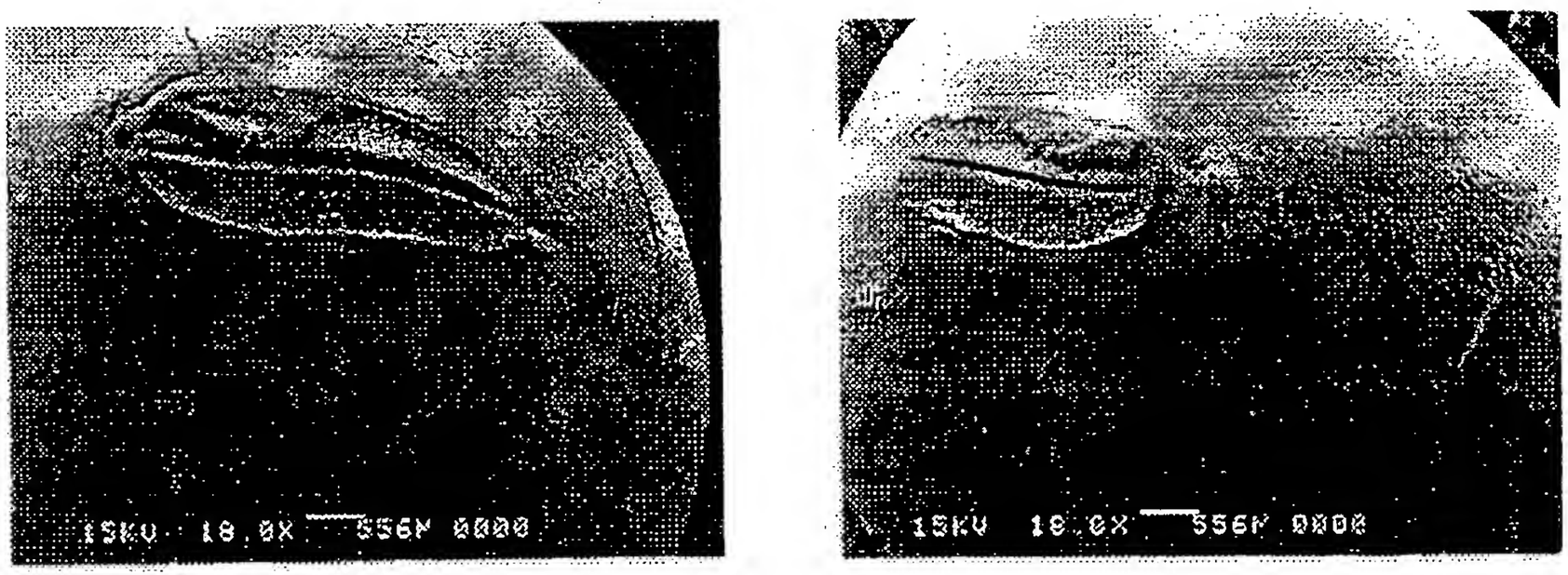


FIG. 16A

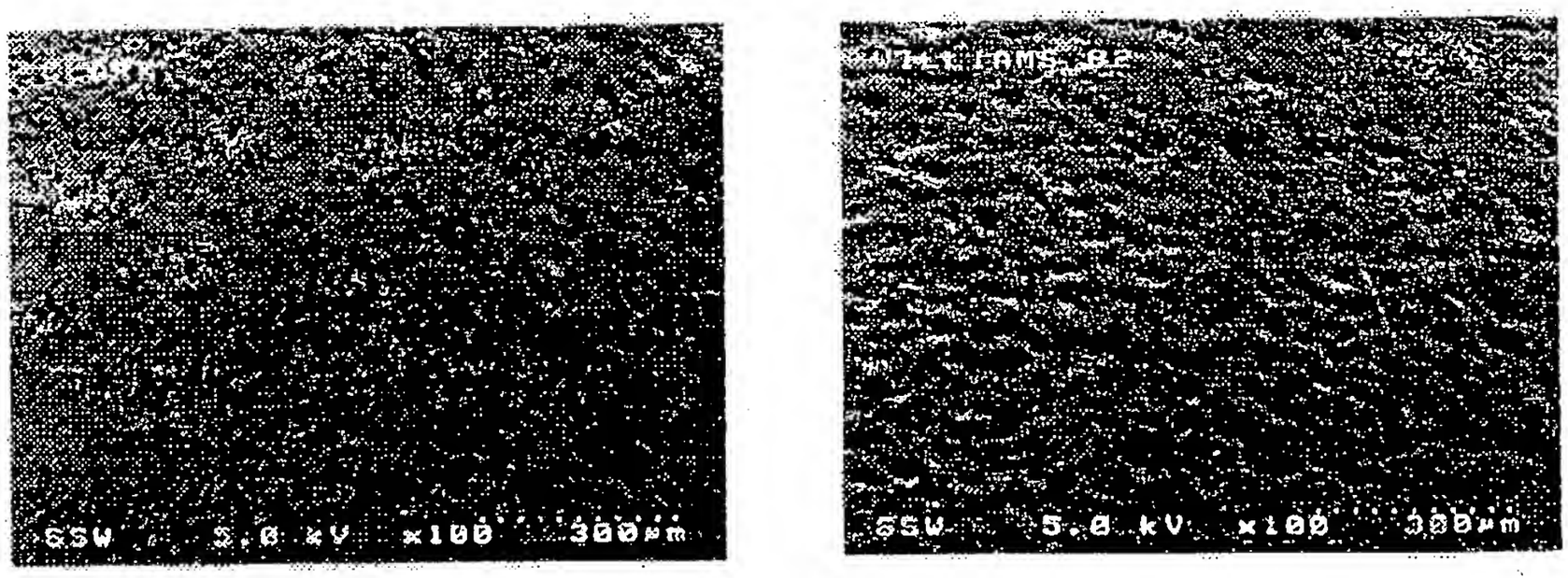


FIG. 16B

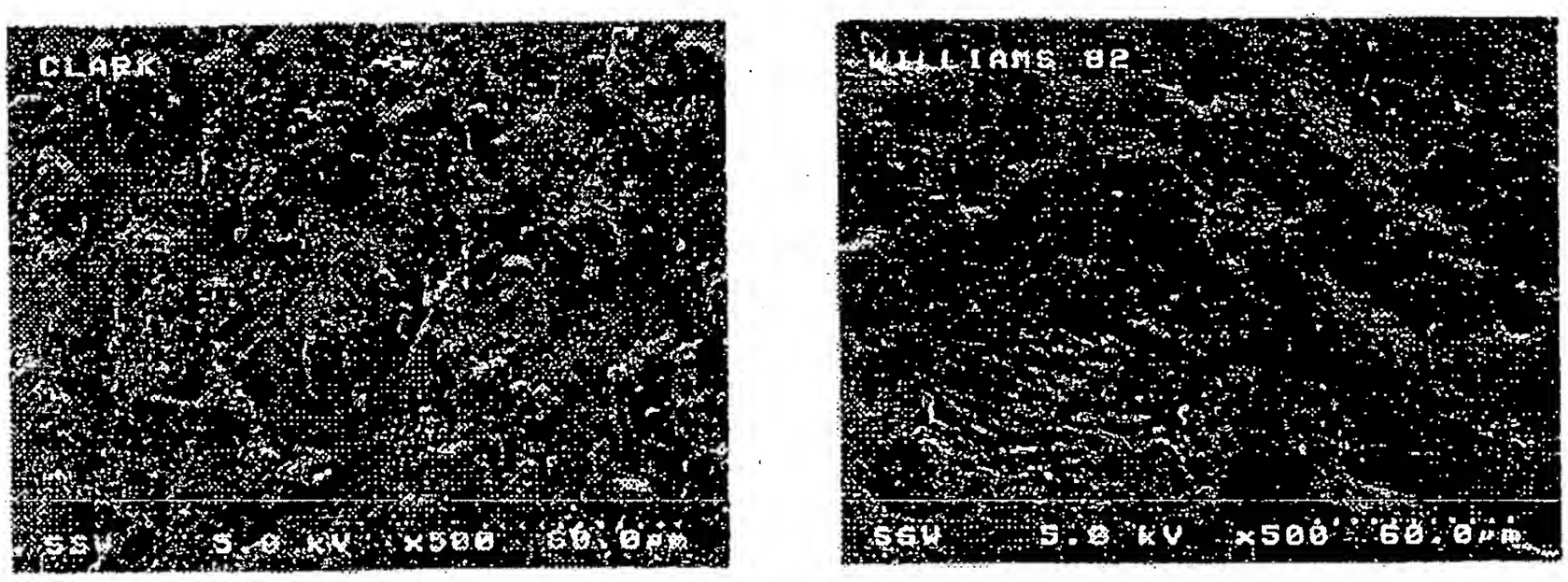


FIG. 16C

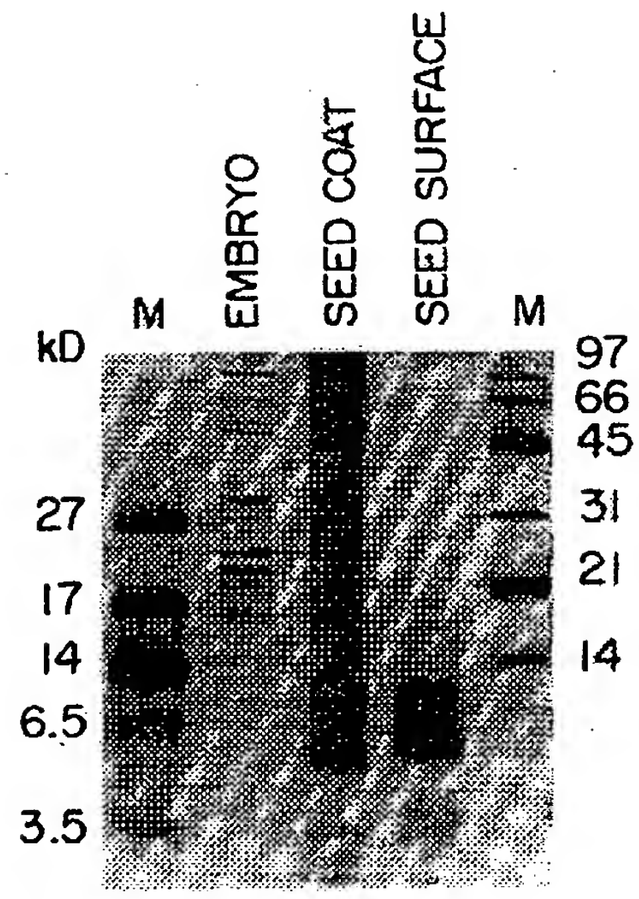


FIG. 17A

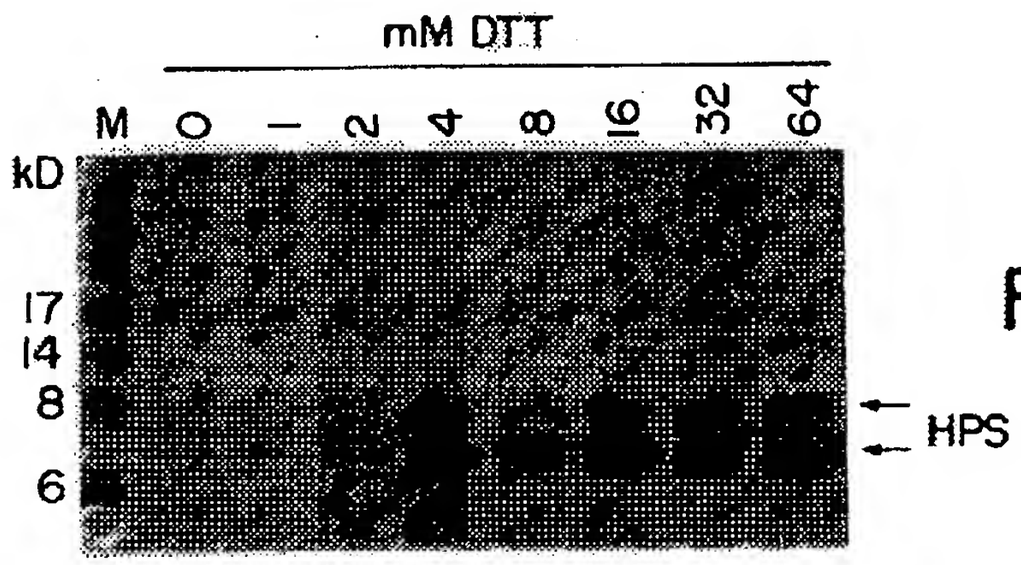


FIG. 17B

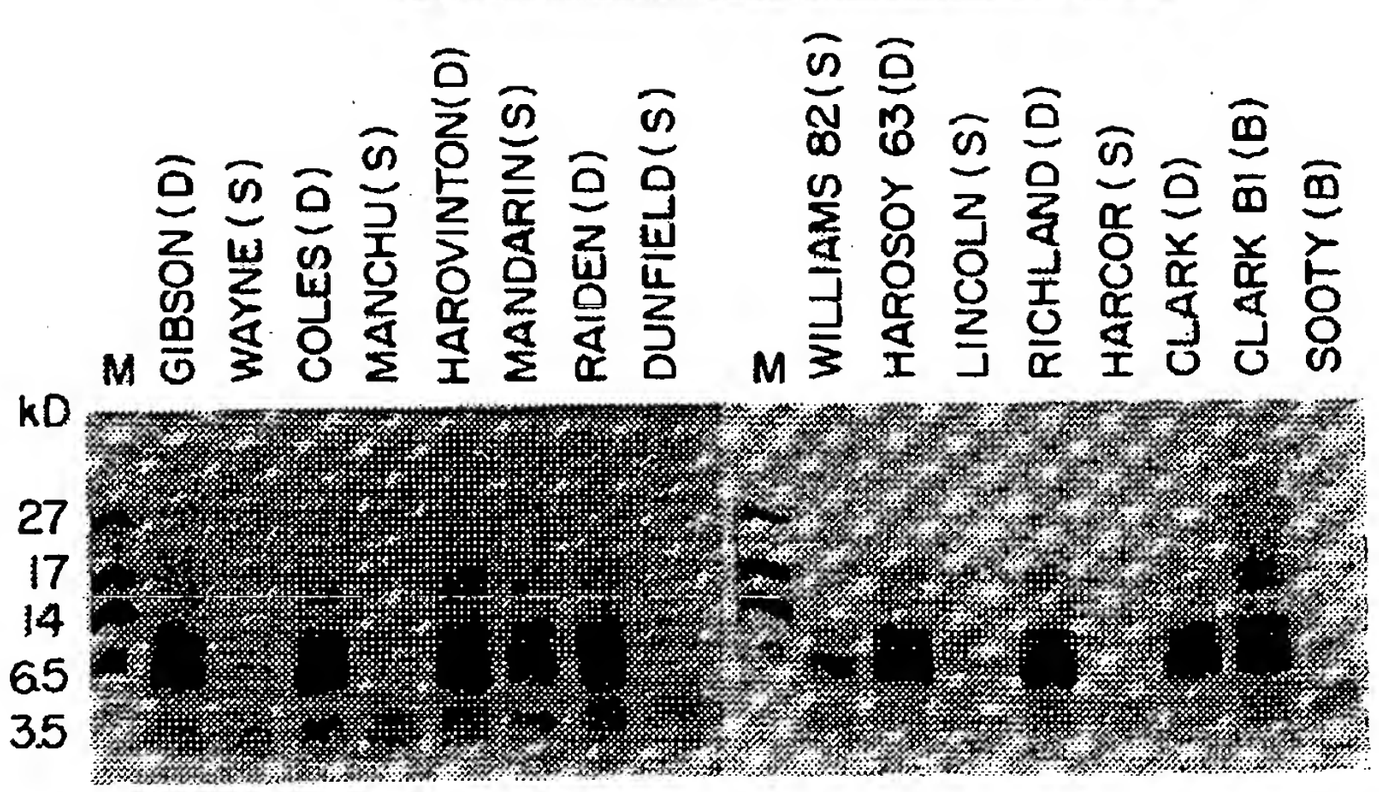


FIG. 17C

WEST

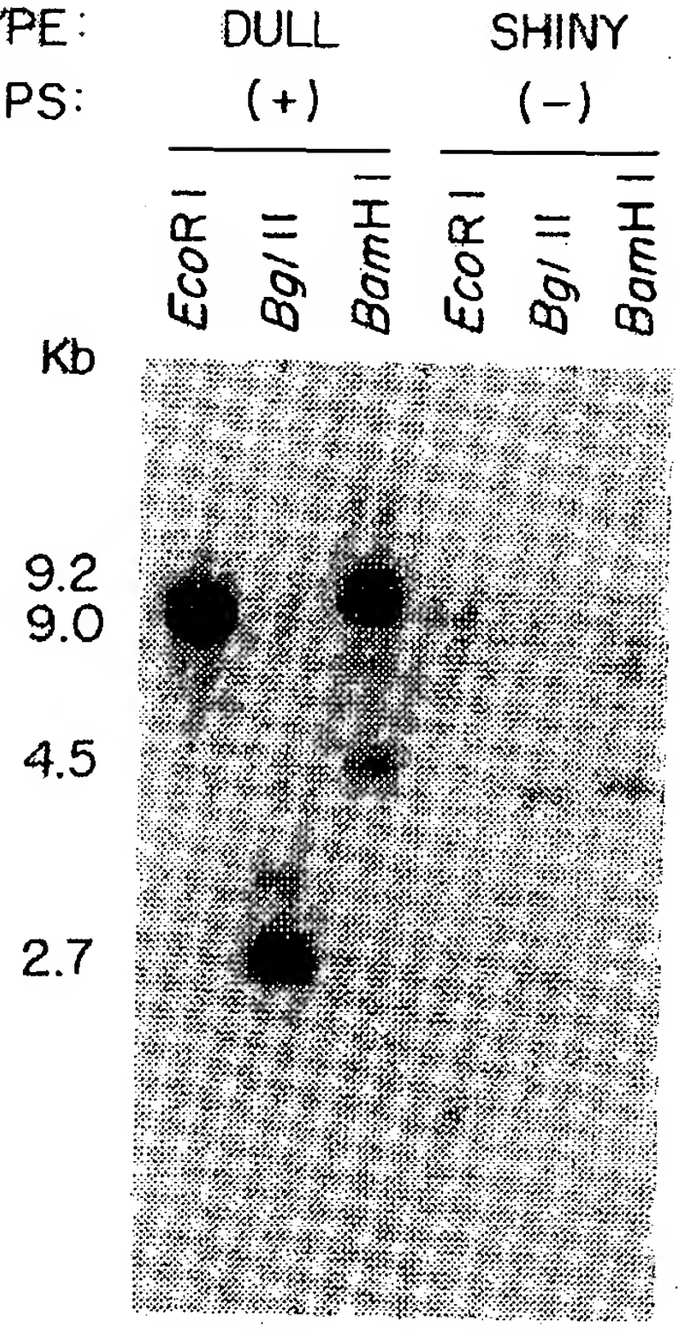

PHENOTYPE:
DULL
SHINY
HPS:
(+)
(−)
EcoR I
Bgl II
BamH I
EcoR I
Bgl II
BamH I
Kb
9.2
9.0
4.5
2.7

FIG. 18

```
--C AAT GCT GCG TTA ACT CCT AGA CAT TAC TGG GAA ACG ATG CTT CCA AGA ACT CCC   55
    N   A   A   L   T   P   R   H   Y   W   E   T   M   L   P   R   T   P   18

TTG CCG AAA GCA ATC ACA GAG CTA CTA AGC CTT GAA AGT AGG TCC ATA TTT GAA TAT  112
 L   P   K   A   I   T   E   L   L   S   L   E   S   R   S   I   F   E   Y   37

GCC GGG AAT GAT GAC CAG TCA GAA AGT AGG TCC ATA TTA GGA TAC GCT GGC TAT AAT  169
 A   G   N   D   D   Q   S   E   S   R   S   I   L   G   Y   A   G   Y   N   56

CAA GAC GAG GAT GAT GTG AGC AAA CAC AAT ATA CAA ATC TTC AAC AGG TTG TTT TTC  226
 Q   D   E   D   D   V   S   K   H   N   I   Q   I   F   N   R   L   F   F   75

TTG GAA GAG GAC CTG CGT GCT GGC AAA ATA TTC AAC ATG AAG TTC GTC AAC AAC ACA  283
 L   E   E   D   L   R   A   G   K   I   F   N   M   K   F   V   N   N   T   94

AAA GCC ACA GTC CCG TTG CTA CCG CGC CAA ATT TCG AAA CAA ATA CCG TTC TCA GAA  340
 K   A   T   V   P   L   L   P   R   Q   I   S   K   Q   I   P   F   S   E  113

GAT AAA AAG AAG CAA GTG TTG GCG ATG CTT GGC GTG GAA GCG AAC TCA AGC AAC GCC  397
 D   K   K   K   Q   V   L   A   M   L   G   V   E   A   N   S   S   N   A  132

AAG ATC ATA GCG GAG ACC ATT GGT CTT TGC CAA GAG CCT GCA ACG GAG GGA GAA AGG  454
 K   I   I   A   E   T   I   G   L   C   Q   E   P   A   T   E   G   E   R  151

AAA CAC TGC GCG ACT TCG TTG GAG TCC ATG GTT GAT TTC GTC GTT TCC GCG CTC GGG  511
 K   H   C   A   T   S   L   E   S   M   V   D   F   V   V   S   A   L   G  170

AAG AAC GTT GGT GCT TTC TCA ACA GAG AAA GAA AGG GAA ACT GAG TCT GGA AAG TTT  568
 K   N   V   G   A   F   S   T   E   K   E   R   E   T   E   S   G   K   F  189

GTA GTG GTG AAA AAT GGG GTG AGG AAG TTG GGA GAT GAT AAG GTT ATT GCC TGT CAT  625
 V   V   V   K   N   G   V   R   K   L   G   D   D   K   V   I   A   C   H  208

CCA ATG AGT TAC CCT TAT GTT GTG TTT GGG TGT CAT CTA GTG CCA AGG AGT AGC GGG  682
 P   M   S   Y   P   Y   V   V   F   G   C   H   L   V   P   R   S   S   G  227

TAT TTG GTG CGC TTG AAG GGA GAA GAT GGG GTT CGA GTG AAA GCA GTT GTT GCG TGC  739
 Y   L   V   R   L   K   G   E   D   G   V   R   V   K   A   V   V   A   C  246

CAC AGA GAC ACG TCA AAG TGG GAC CAT AAT CAT GGG GCA TTC AAA GTG CTC AAT CTT  796
 H   R   D   T   S   K   W   D   H   N   H   G   A   F   K   V   L   N   L  265

AAG CCT GGG AAT GGT ACA GTA TGC CAT GTC TTC ACT GAG GGG AAT CTT CTT TGG CTT  853
 K   P   G   N   G   T   V   C   H   V   F   T   E   G   N   L   L   W   L  284

CCA AAT TAG attaattaccatatacatatttgtccttgttctatccttaaataagtggaatcacctgaagaa  925
 P   N   *                                                                  286

ttgtgcgtaatgagttgtttgtctttgtggaaattgttatctgtcttgcatcaccaaataggtatatataaaata 1000

acaggagcgtggtatttgttgcacaaaaatggatttcaaccgatcaaaaaaatatagcctttaccaattagaagg 1075

gtttggctttgttagcaataataaaaataaaatatcttgatgg(a)n                            1119
```

FIG. 19(A)

A

```
SC4c        FFLEEDLRAG KIFNMKFVNN --TKATVPLL PRQISKQIPF SEDKKKQVLA MLGVEANSSN 131
RD22        FFLEKDLVRG KEMNVRFNAE DGYGGKTAFL PRGEAETVPF GSEKFSETLK RFSVEAGSEE 235
PG1B        FFREKMLKSG TIMPMPDIK- -DKMPKRSFL PRVIASKLPF STSKIAELKK IFHAGDESQV 472
Sali3-2     FFYKEDLHPG KTMKVQFTKR ---------- --------PY AQPY--GVYT WLTDIKDTSK 215
USP         FF-EHDLHPG KNFNLGHTNS VGSIIR---- --------PF TKSR--QGVT --DSIWLANK 111
ADR6        FFYKEDLHPG KTMKVQFSKP ---------- --------PF QQPW--GVGT WLKEIKDTTK 111

SC4c        AKIIAETIGL CQE-PATEGE RKHCATSLES MVDFVVSALG KN-VGAFSTE KERETESGK- 188
RD22        AEMMKKTIEE C-EARKVSGE EKYCATSLES MVDFSVSKLG KYHVRAVSTE VAKKNAPMQK 294
PG1B        EKMIGDALSE C-ERAPSAGE TKRCVNSAED MIDFATSVLG RN-VVVRTTE DTKGSNGNIM 530
Sali3-2     EGYSFEEI-- CIKKEAFEGE EKFCAKSLGT VIGFAISKLG KN-IQVLSSS FVNKQE---- 168
USP         EKQSLEDF-- CYSPTAI-AE HKHCVSSLKS MIDQVISHFG STKIKAISSN FAPYQD---- 164
ADR6        EGYSFEEL-- CIKKEAIEGE EKFCAKSLGT VIGFAISKLG KN-IQVLSSS FVNKQD---- 164

SC4c        FVVVKNGVRK LGDDKVIACH PMSYPYVVFG CHLVPR-SSG YLVRLKGEDG VR-VKAVVAC 246
RD22        YKIAAAGVKK LSDDKSVVCH KQKYPFAVFY CHKAMM-TTV YAVPLEGENG MR-AKAVAVC 352
PG1B        I-GSVKGING GKVTKSVSCH QTLYPYLLYY CHSVPKVRVY EADILDPNSK VKINHGVAIC 589
Sali3-2     -QYTVEGVQN LG-DKAVMCH GLNFRTAVFY CHKV-RETTA FVVPLVAGDG TK-TQALAVC 224
USP         -QYVVEDVKK VG-DNAVMCH RLNFEKVVFN CHQV-RDTTA YVVSLVASDG TK-TKALTVC 220
ADR6        -QYTVEGVQN LG-DKAVMCH RLNFRTAVFY CHEV-RETTA FMVPLVAGDG TK-TQALAIC 220

SC4c        HRDTSKWDHN HGAFKVLNLK PGNGTVCHVF TEGNLLWLPN *                  286
RD22        HKNTSAWNPN HLAFKVLKVK PGTVPVCHFL PETHVVWFSY *                  392
PG1B        HVDTSSWGPS HGAFVALGSG PGKIEVCHWI FENDMTWAIA D*                       630
Sali3-2     HSDTSGMNH- HILHELMGVD PGTNPVCHFL GSKAILWVPN ISMDTAYQTN VVV*    276
USP         HHDTRGMNP- ELLYEALEVT PGTVPVCHFI GNKAAAWVPN HTADNLCVM*              268
ADR6        HSNTSGMNH- QMLHQLMGVD PGTNPVCHFL GSKAILWVPN LSVDTAYQTN IVA*    272
```

B

```
SC4c        --NAALTPRL YWETMLPRTP LPKAITELLS L  29
RD22        AIAADLTPER YWSTALPNTP IPNSLHNLLT F  48
Sali3-2     HVHASLPEED YWEAVWPNTP IPTALRDVLK P  53
USP         GITATSSGED YWQSIWPNTP LPKTFSDLLI P  48
ADR6        ARESHARDED FWHAVWPNTP IPSSLRDLLK P  49
```

FIG. 19(B)

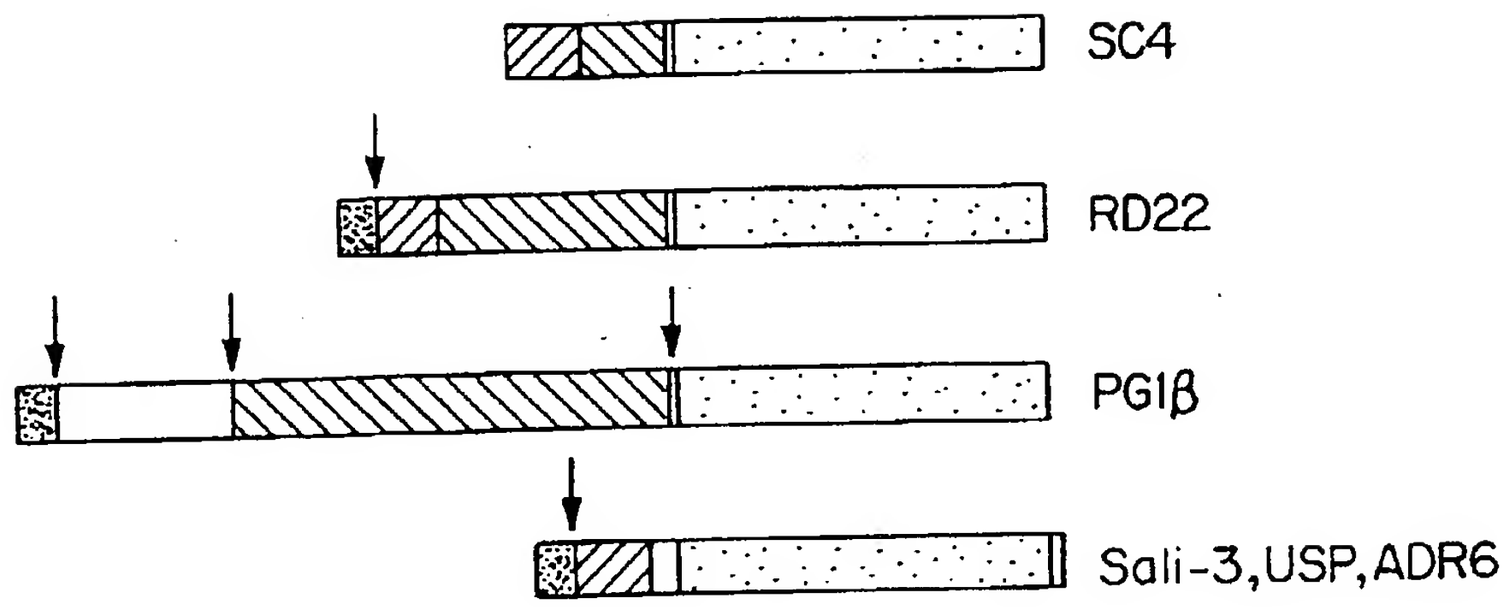

SC4
RD22
PG1β
Sali-3,USP,ADR6
100aa
HYDROPHOBIC REGION:
CONSERVED SEGMENT:
REPEATED REGION:
BURP DOMAIN:
PEPTIDE CLEAVAGE POINT:

FIG. 19C

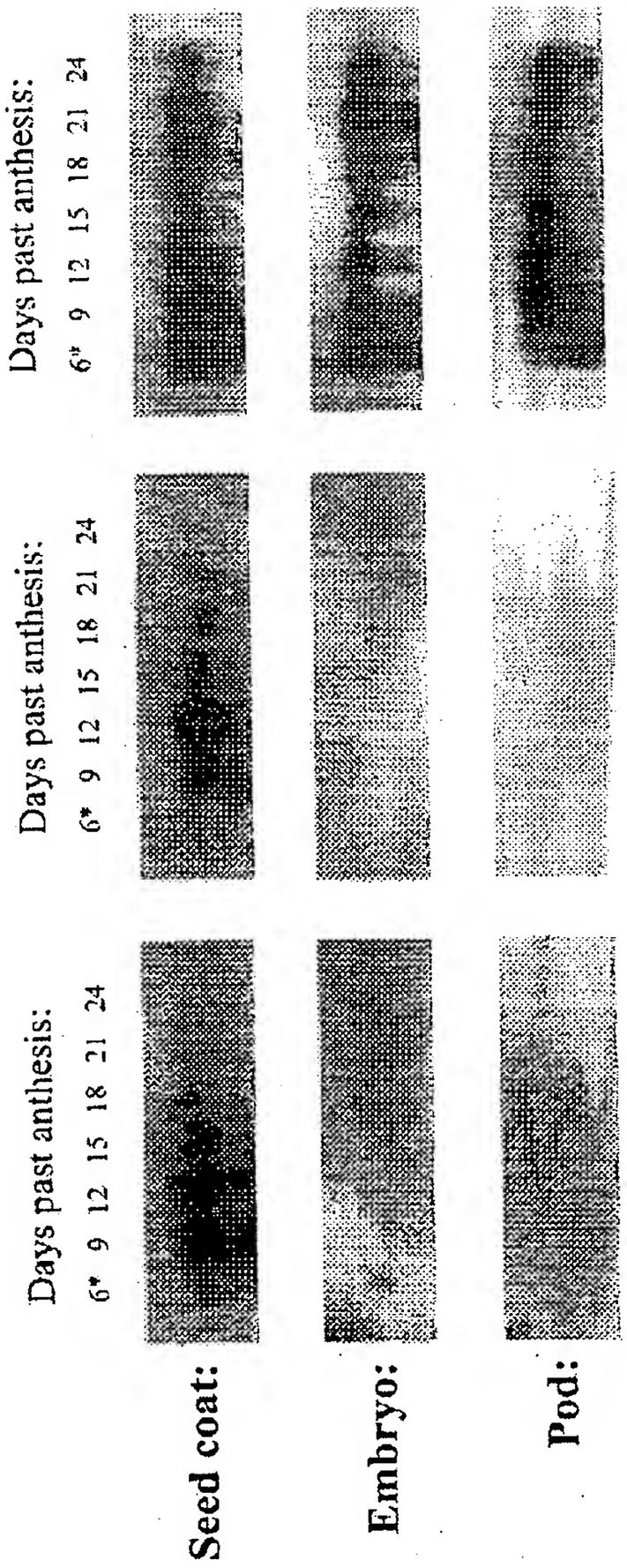


Fig. 20 A

Fig. 20 B

Fig. 20 C

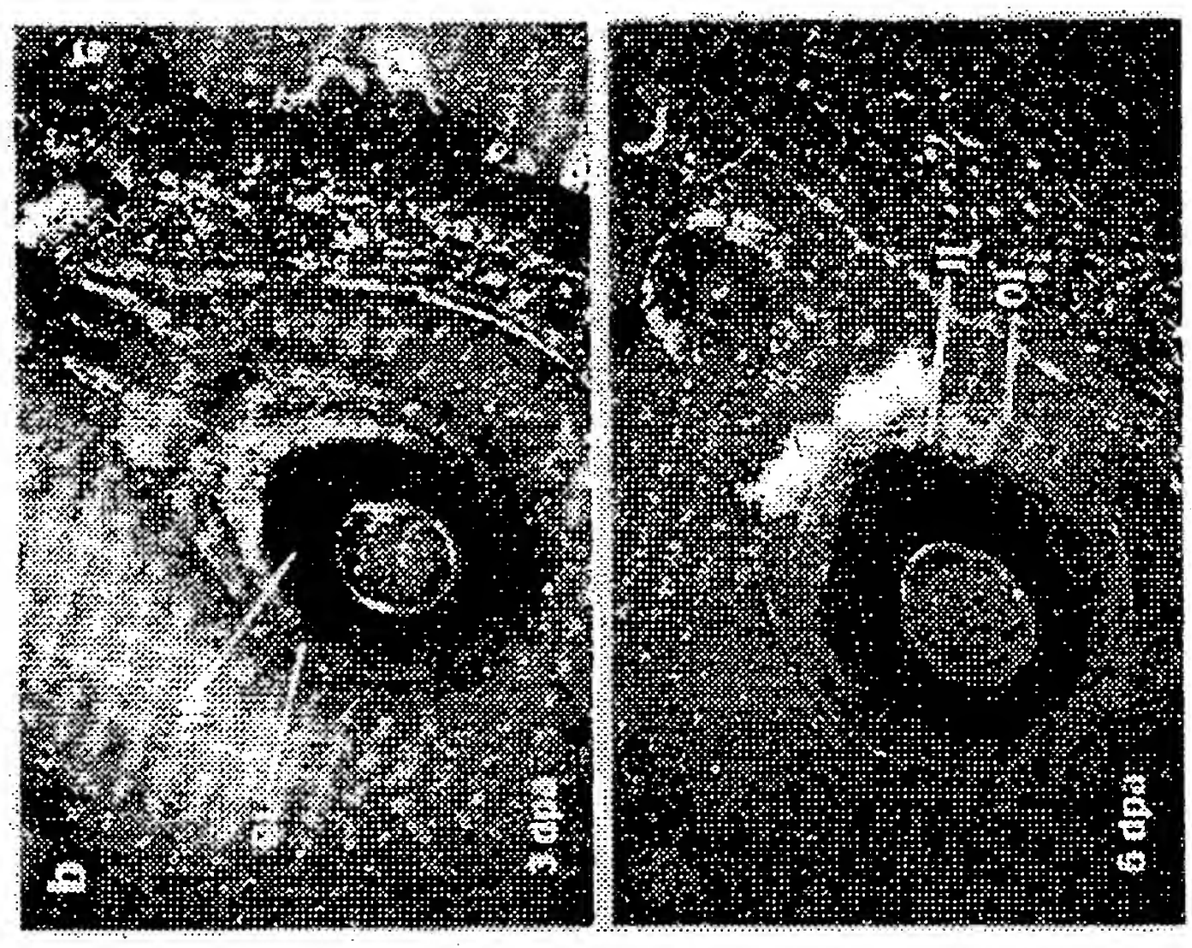


FIG. 21B

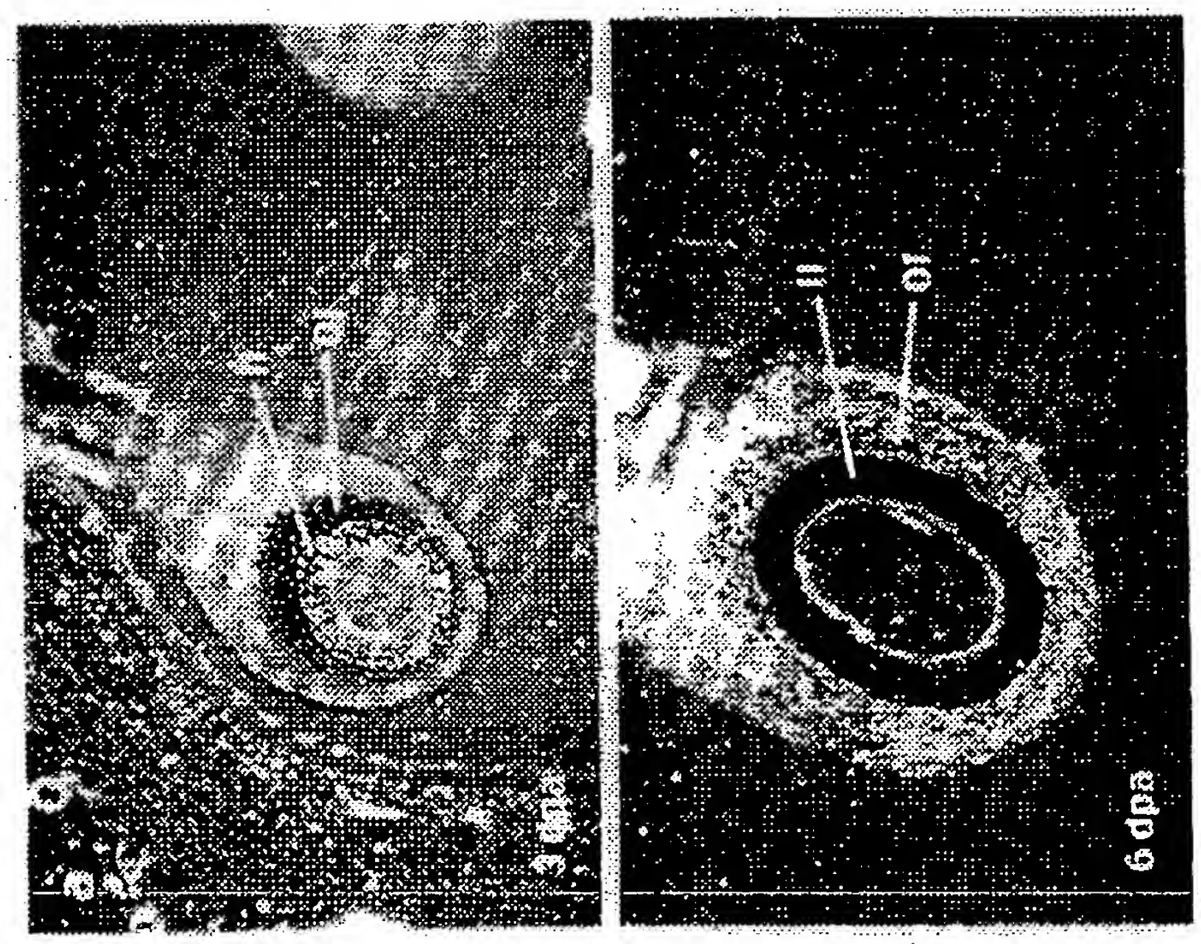


FIG. 21A

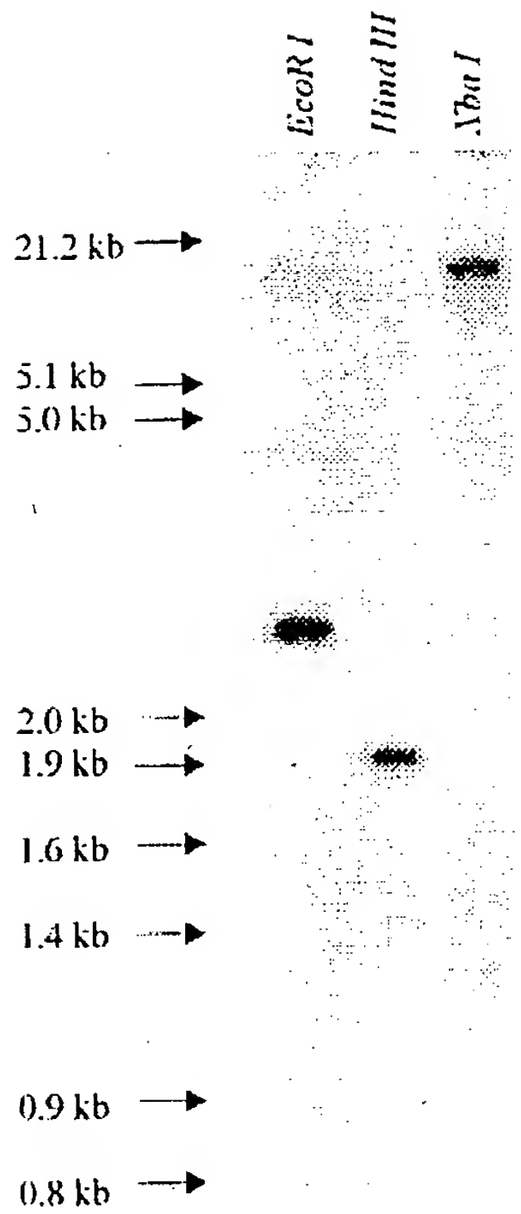

EcoR I
Hind III
Xba I
21.2 kb
5.1 kb
5.0 kb
2.0 kb
1.9 kb
1.6 kb
1.4 kb
0.9 kb
0.8 kb

Fig. 22 A

WEST

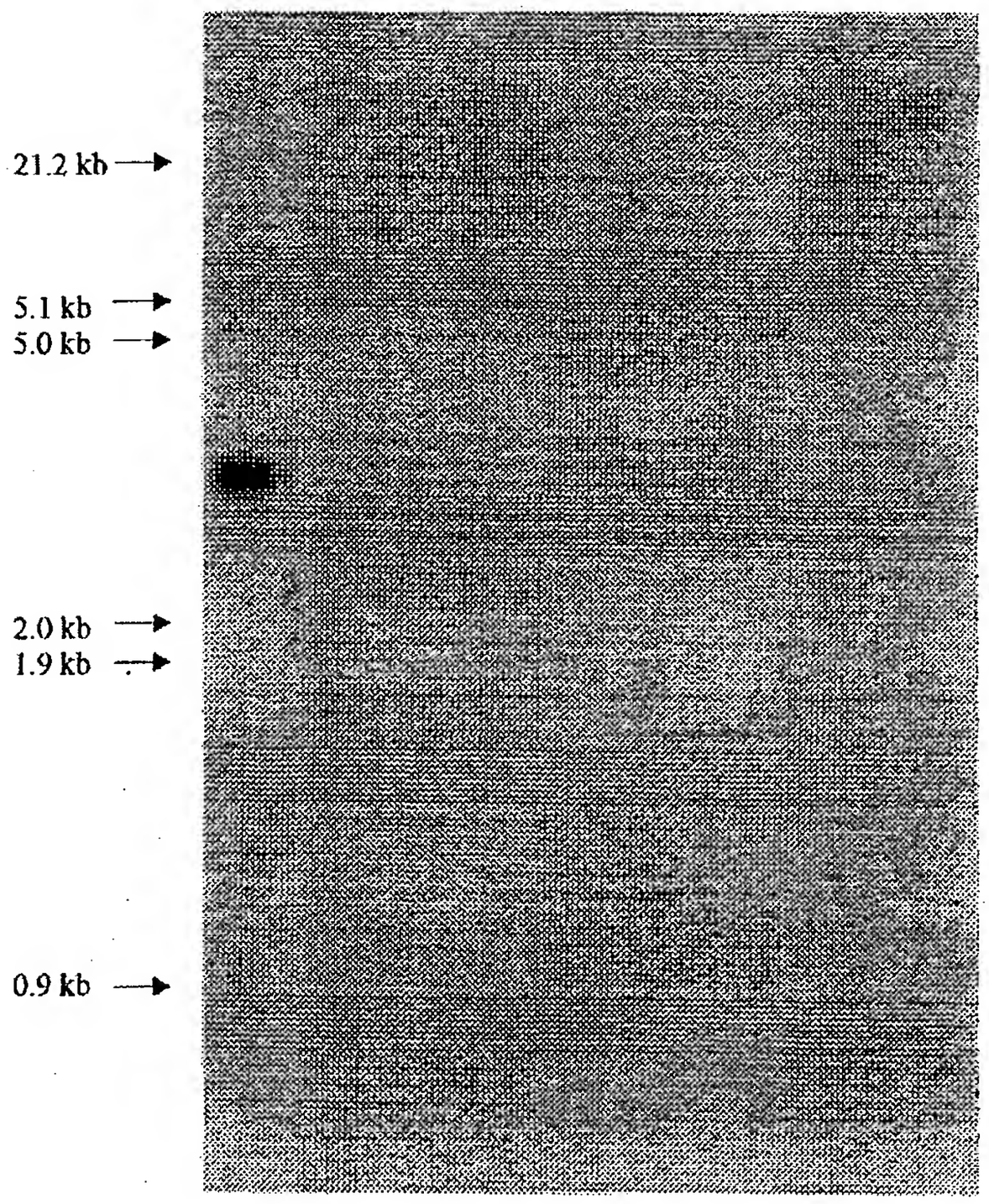

soybean
pea
canola
oat (ogle)
oat (kanota)
onion
pepper
mimosa
black spruce
birch
21.2 kb
5.1 kb
5.0 kb
2.0 kb
1.9 kb
0.9 kb

Fig. 22 B

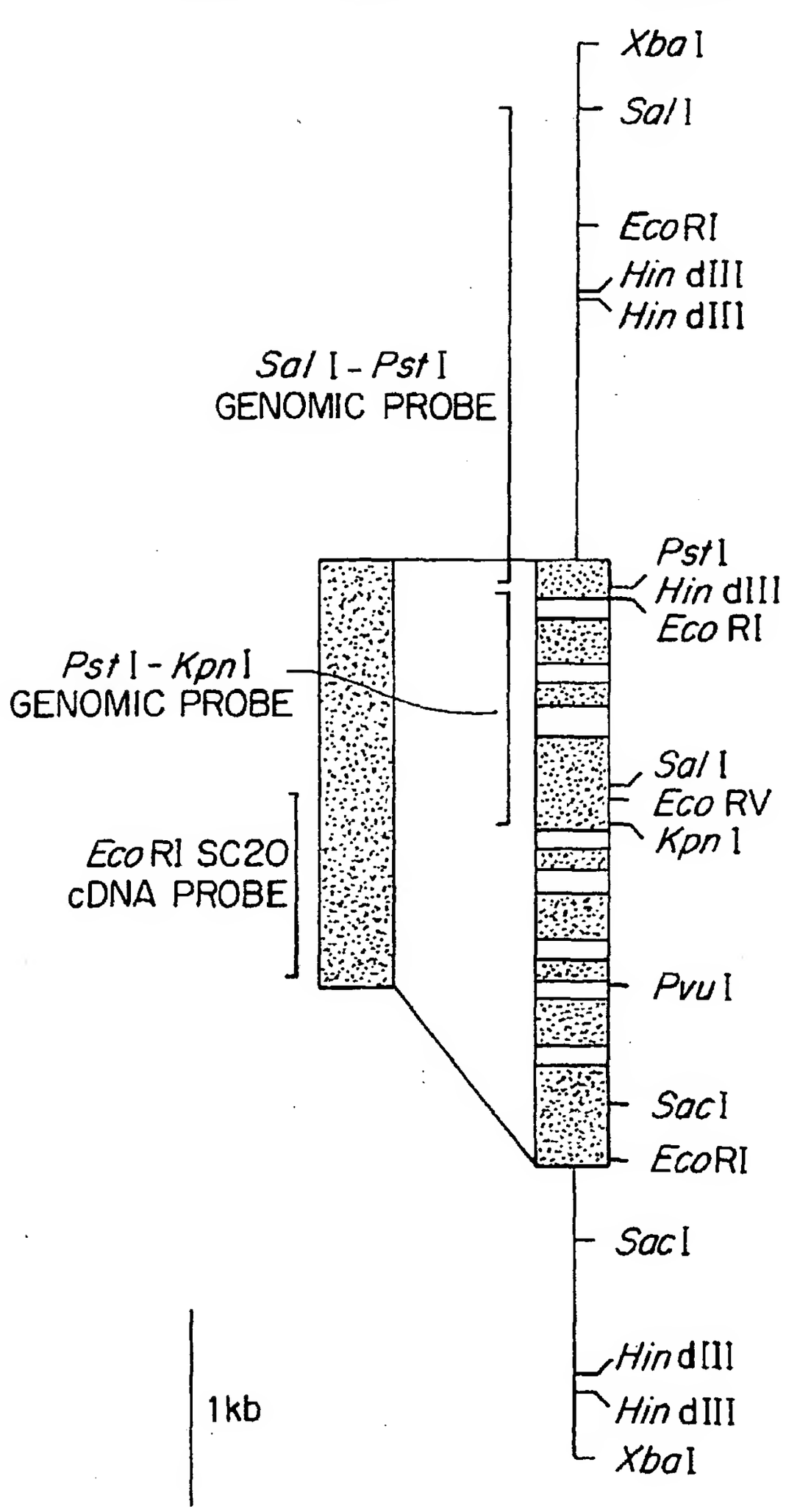

SC20
(cDNA CLONE):
sc20
(GENOMIC CLONE):
Xba I
Sal I
EcoRI
Hin dIII
Hin dIII
Sal I - Pst I
GENOMIC PROBE
Pst I
Hin dIII
Eco RI
Pst I - Kpn I
GENOMIC PROBE
Sal I
Eco RV
Kpn I
EcoRI SC20
cDNA PROBE
Pvu I
Sac I
EcoRI
Sac I
Hin dIII
Hin dIII
Xba I
1kb

FIG. 23A

```
caaagttttaac ATG AAA GGC AAT AAT ACA CTT TTG TTG CAT TTA TTC TAC ACT ACT CTC  60
             M   K   G   N   N   T   L   L   L   H   L   F   Y   T   T   L   16

TTC CTG TTT CTT GTA GTG TCA AGT TCA TCT TCA ACA GGG AAT GAA AGT AAC GAT GAC  117
 F   L   F   L   V   V   S   S   S   S   S   T   G   N   E   S   N   D   D   35

ACT AAC AGT AAA GAA GTT TAT ATC GTG TAC ATG GGA GCT GCA GAT TCA ACA AAA GCT  174
 T   N   S   K   E   V   Y   I   V   Y   M   G   A   A   D   S   T   K   A   54

TCT CTT AAA AAT GAG CAC GCT CAG ATT CTG AAT TCA GTG CTA AGA AGG AAT GAG AAT  231
 S   L   K   N   E   H   A   Q   I   L   N   S   V   L   R   R   N   E   N   73

GCC CTA GTA CGG AAC TAC AAG CAT GGT TTC TCT GGG TTC GCA GCT CGT CTA TCA AAA  288
 A   L   V   R   N   Y   K   H   G   F   S   G   F   A   A   R   L   S   K   92

GAG GAG GCA AAC TCA ATT GCT CAG AAA CCT GGT GTG GTG TCT GTT TTC CCT GAC CCC  345
 E   E   A   N   S   I   A   Q   K   P   G   V   V   S   V   F   P   D   P   111

ATT CTG AAG CTC CAC ACT ACA CGT TCA TGG GAT TTC CTC AAA AGC CAA ACT CGT GTC  402
 I   L   K   L   H   T   T   R   S   W   D   F   L   K   S   Q   T   R   V   130

AAT ATC GAC ACC AAA CCA AAT ACG CTG TCC GGT TCT TCT TTT TCT TCA TCA GAC GTC  459
 N   I   D   T   K   P   N   T   L   S   G   S   S   F   S   S   S   D   V   149

ATT CTT GGC GTC TTA GAC ACA GGC ATA TGG CCA GAG GCG GCG AGT TTT AGC GAC AAG  516
 I   L   G   V   L   D   T   G   I   W   P   E   A   A   S   F   S   D   K   168

GGT TTC GGT CCT GTT CCA TCC CGA TGG AAA GGC ACC TGC ATG ACA TCA AAA GAC TTC  573
 G   F   G   P   V   P   S   R   W   K   G   T   C   M   T   S   K   D   F   187

AAT TCC TCT TGT TGT AAC AGG AAG ATA ATT GGC GCG AGG TTT TAC CCT AAC CCA GAG  630
 N   S   S   C   C   N   R   K   I   I   G   A   R   F   Y   P   N   P   E   206

GAG AAA ACG GCA AGG GAT TTC AAC GGA CAT GGG ACT CAC GTT TCG TCG ACG GCA GTG  687
 E   K   T   A   R   D   F   N   G   H   G   T   H   V   S   S   T   A   V   225

GGC GTG CCG GTG AGT GGC GCA TCG TTC TAT GGT CTG GCG GCG GGG ACG GCA AGG GGT  744
 G   V   P   V   S   G   A   S   F   Y   G   L   A   A   G   T   A   R   G   244

GGG TCC CCT GAG TCA AGG TTG GCG GTT TAC AAA GTG TGT GGG GCT TTT GGG TCA TGT  801
 G   S   P   E   S   R   L   A   V   Y   K   V   C   G   A   F   G   S   C   263

CCT GGG TCG GCC ATT CTT GCG GGG TTT GAC GAT GCC ATT CAC GAC GGA GTG GAT ATC  858
 P   G   S   A   I   L   A   G   F   D   D   A   I   H   D   G   V   D   I   282

TTG TCG CTG TCG CTC GGT GGA TTC GGT GGA ACC AAA ACC GAT TTG ACC ACC GAC CCG  915
 L   S   L   S   L   G   G   F   G   G   T   K   T   D   L   T   T   D   P   301

ATT GCG ATT GGA GCA TTC CAC TCC GTC CAG CGC GGC ATC CTG GTG GTC TGC GCC GCC  972
 I   A   I   G   A   F   H   S   V   Q   R   G   I   L   V   V   C   A   A   320

GGG AAC GAC GGA GAA CCA TTC ACC GTT CTC AAC GAC GCA CCT TGG ATT TTA ACC GTT 1029
 G   N   D   G   E   P   F   T   V   L   N   D   A   P   W   I   L   T   V   339

GCA GCT TCC ACC ATC GAC CGT GAT CTT CAA TCC GAC GTG GTC TTG GGT AAT AAC CAA 1086
 A   A   S   T   I   D   R   D   L   Q   S   D   V   V   L   G   N   N   Q   358
```

FIG. 23(B)

```
GTC GTC AAG GGA AGA GCC ATA AAT TTC TCC CCT CTT TTA AAT TCT CCC GAT TAT CCA 1143
 V   V   K   G   R   A   I   N   F   S   P   L   L   N   S   P   D   Y   P  377

ATG ATA TAT GCT GAG TCT GCT GCC AGG GCA AAT ATC TCC AAC ATA ACT GAT GCA AGA 1200
 M   I   Y   A   E   S   A   A   R   A   N   I   S   N   I   T   D   A   R  396

CAA TGC CAC CCA GAT TCA TTA GAT CCA AAA AAA GTC ATA GGG AAG ATT GTG GTT TGT 1257
 Q   C   H   P   D   S   L   D   P   K   K   V   I   G   K   I   V   V   C  415

GAT GGA AAA AAT GAC ATT TAT TAT TCA ACT GAT GAG AAA ATT GTC ATA GTG AAG GCG 1314
 D   G   K   N   D   I   Y   Y   S   T   D   E   K   I   V   I   V   K   A  434

TTG GGA GGA ATA GGT CTG GTT CAT ATT ACT GAT CAA TCT GGA TCA GTA GCA TTT TAT 1371
 L   G   G   I   G   L   V   H   I   T   D   Q   S   G   S   V   A   F   Y  453

TAT GTG GAC TTC CCA GTA ACA GAG GTA AAA TCA AAA CAT GGC GAC GCA ATC CTC CAG 1428
 Y   V   D   F   P   V   T   E   V   K   S   K   H   G   D   A   I   L   Q  472

TAC ATC AAC TCA ACT AGC CAT CCA GTG GGA ACA ATA CTA GCA ACA GTT ACA ATT CCT 1485
 Y   I   N   S   T   S   H   P   V   G   T   I   L   A   T   V   T   I   P  491

GAT TAT AAG CCT GCT CCC CGG GTG GGT TAT TTT TCA TCA AGA GGG CCT TCA TTG ATT 1542
 D   Y   K   P   A   P   R   V   G   Y   F   S   S   R   G   P   S   L   I  510

ACA AGC AAT GTT CTC AAG CCT GAT ATT GCA GCC CCG GGA GTT AAC ATT CTC GCT GCA 1599
 T   S   N   V   L   K   P   D   I   A   A   P   G   V   N   I   L   A   A  529

TGG TTT GGA AAT GAC ACA TCA GAG GTT CCA AAA GGA AGA AAG CCC TCA CTA TAT CGC 1656
 W   F   G   N   D   T   S   E   V   P   K   G   R   K   P   S   L   Y   R  548

ATA CTC TCA GGA ACT TCC ATG GCT ACT CCA CAT GTT TCA GGG CTT GCA TGC AGT GTC 1713
 I   L   S   G   T   S   M   A   T   P   H   V   S   G   L   A   C   S   V  567

AAA AGA AAA AAC CCC ACT TGG AGT GCC TCC GCA ATC AAA TCT GCC ATC ATG ACT TCA 1770
 K   R   K   N   P   T   W   S   A   S   A   I   K   S   A   I   M   T   S  586

GCA ATT CAA AAT GAC AAT TTG AAG GGT CCC ATA ACA ACG GAT TCA GGG TTG ATA GCC 1827
 A   I   Q   N   D   N   L   K   G   P   I   T   T   D   S   G   L   I   A  605

ACA CCT TAT GAC TAT GGA GCA GGG GCA ATT ACA ACA TCT GAA CCA TTG CAA CCG GGG 1884
 T   P   Y   D   Y   G   A   G   A   I   T   T   S   E   P   L   Q   P   G  624

CTA GTT TAT GAA ACC AAC AAC GTT GAC TAC TTG AAC TAT TTG TGT TAC AAT GGA CTT 1941
 L   V   Y   E   T   N   N   V   D   Y   L   N   Y   L   C   Y   N   G   L  643

AAC ATA ACC ATG ATA AAG GTC ATC TCC GGA ACT GTC CCC GAG AAT TTC AAT TGT CCC 1998
 N   I   T   M   I   K   V   I   S   G   T   V   P   E   N   F   N   C   P  662

AAG GAT TCG AGC TCT GAT CTC ATC TCC AGC ATC AAC TAC CCT TCC ATA GCA GTA AAC 2055
 K   D   S   S   S   D   L   I   S   S   I   N   Y   P   S   I   A   V   N  681

TTC ACT GGC AAA GCA GAC GCG GTC GTG AGT AGA ACT GTC ACA AAC GTT GAC GAA GAA 2112
 F   T   G   K   A   D   A   V   V   S   R   T   V   T   N   V   D   E   E  700

GAT GAA ACA GTG TAC TTC CCC GTT GTT GAA GCT CCT AGT GAA GTA ATT GTC ACA CTC 2169
 D   E   T   V   Y   F   P   V   V   E   A   P   S   E   V   I   V   T   L  719

TTT CCA TAT AAT CTT GAG TTT ACG ACA AGT ATT AAA AAA CAA AGC TAC AAT ATT ACT 2226
 F   P   Y   N   L   E   F   T   T   S   I   K   K   Q   S   Y   N   I   T  738
```

FIG. 23(B)(Cont'd)

```
TTC AGA CCG AAG ACC TCC TTG AAG AAA GAT TTG TTT GGA TCT ATC ACT TGG AGT AAC 2283
 F   R   P   K   T   S   L   K   K   D   L   F   G   S   I   T   W   S   N  757

GAC AAA TAT ATG GTT CGA ATT CCT TTT GTA TTA ACT AAA TAG tgaaattaaaaagtagcga 2344
 D   K   Y   M   V   R   I   P   F   V   L   T   K   *                      770

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FIG. 23(B)(Cont'd)

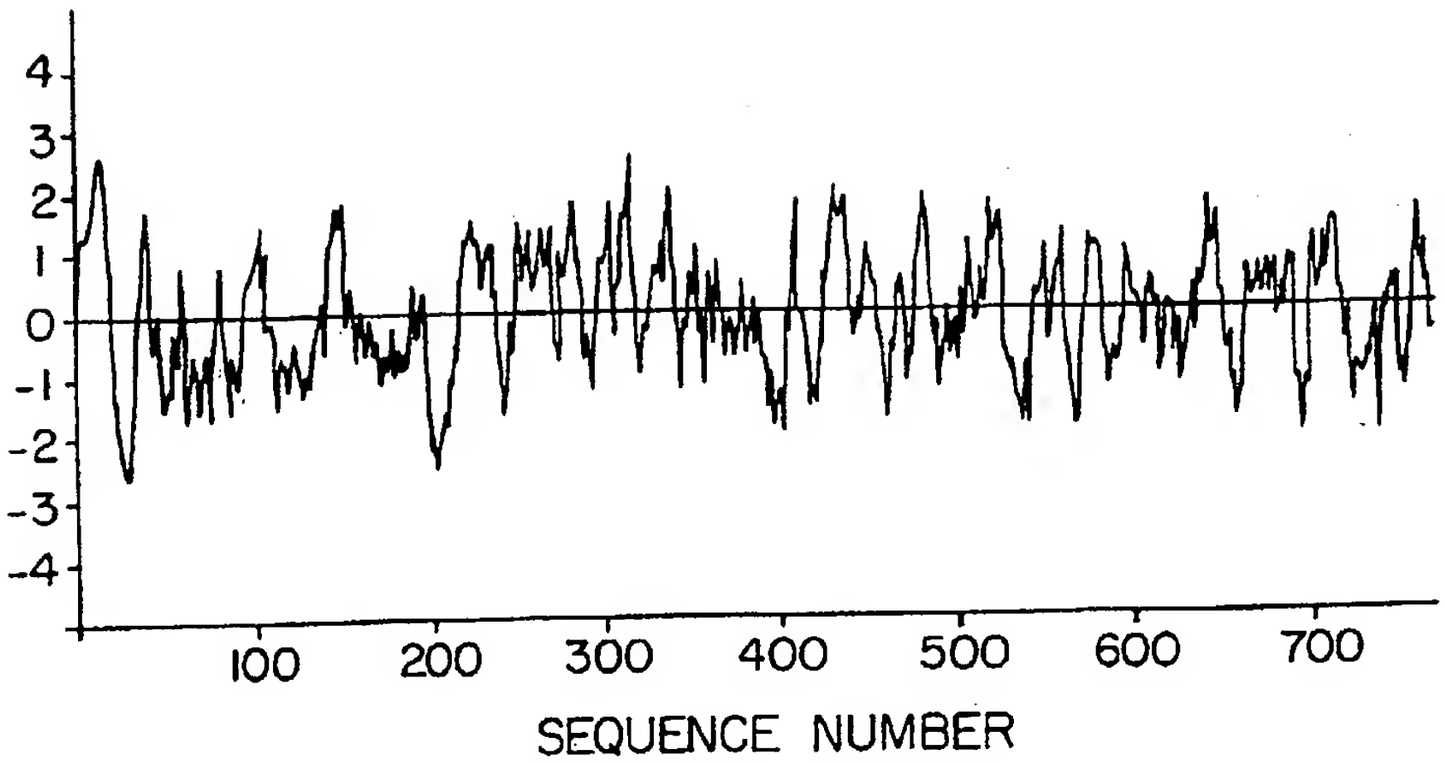

FIG. 23C

D region			H region		
		*			*
SC20:2	SDVILGVLDTGI	156	SC20:2	DFNGHGTHVSSTAVG	224
AF70	TDIILGFLDTGI	145	AF70	DYQGHGTYTAATAAG	229
Cucumisin	SNIVVGVLDTGI	143	Cucumisin	DNTGHGTHTASTAAG	214
P69B	KGVIIGVIDTGI	149	P69B	DDIGHGTHTASTAAG	213
Ag12	EDVIIGVIDSGV	148	Ag12	DTLGHGTHTASTAAG	216
Subtilisin BPNÆ	SNVKVAVIDSGI	142	Subtilisin BPNÆ	DNNSHGTHVAGTVAA	181
Kex2	AGVVAAIVDDGL	178	Kex2	SDDYHGTRCAGEIAA	223
Furin	HGIVVSILDDGI	156	Furin	NDNRHGTRCAGEVAA	204

S region			N region		
		*			#
SC20:2	SGTSMATPHVSGLA	562	SC20:2	SVQRGILVVCAAGNDG	322
AF70	SGTSVAVPHVTGAA	571	AF70	ATQKGILVVSSAGNEG	329
Cucumisin	SGTSMSCPHITGIA	535	Cucumisin	AVERGILTSNSAGNGG	310
P69B	SGTSMSCPHLSGVA	541	P69B	ATERGILVSCSAGNSG	308
Ag12	SGTSMACPHASGVA	547	Ag12	AMEKGVVVSTSAGNAG	318
Subtilisin BPNÆ	NGTSMASPHVAGAA	338	Subtilisin BPNÆ	AVASGVVVVAAAGNEG	264
Kex2	GGTSAAAPLAAGVY	395	Kex2	RDSKGAIYVFASGNGG	316
Furin	TGTSASAPLAAGII	378	Furin	RGGLGSIFVWASGNGG	297

FIG. 23(D)

WEST

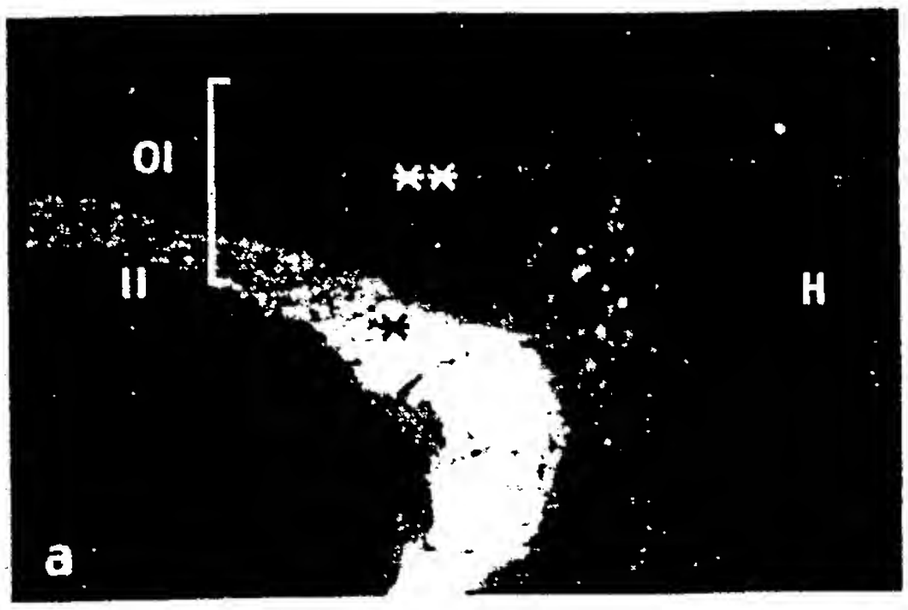

FIG. 24A

FIG. 24B

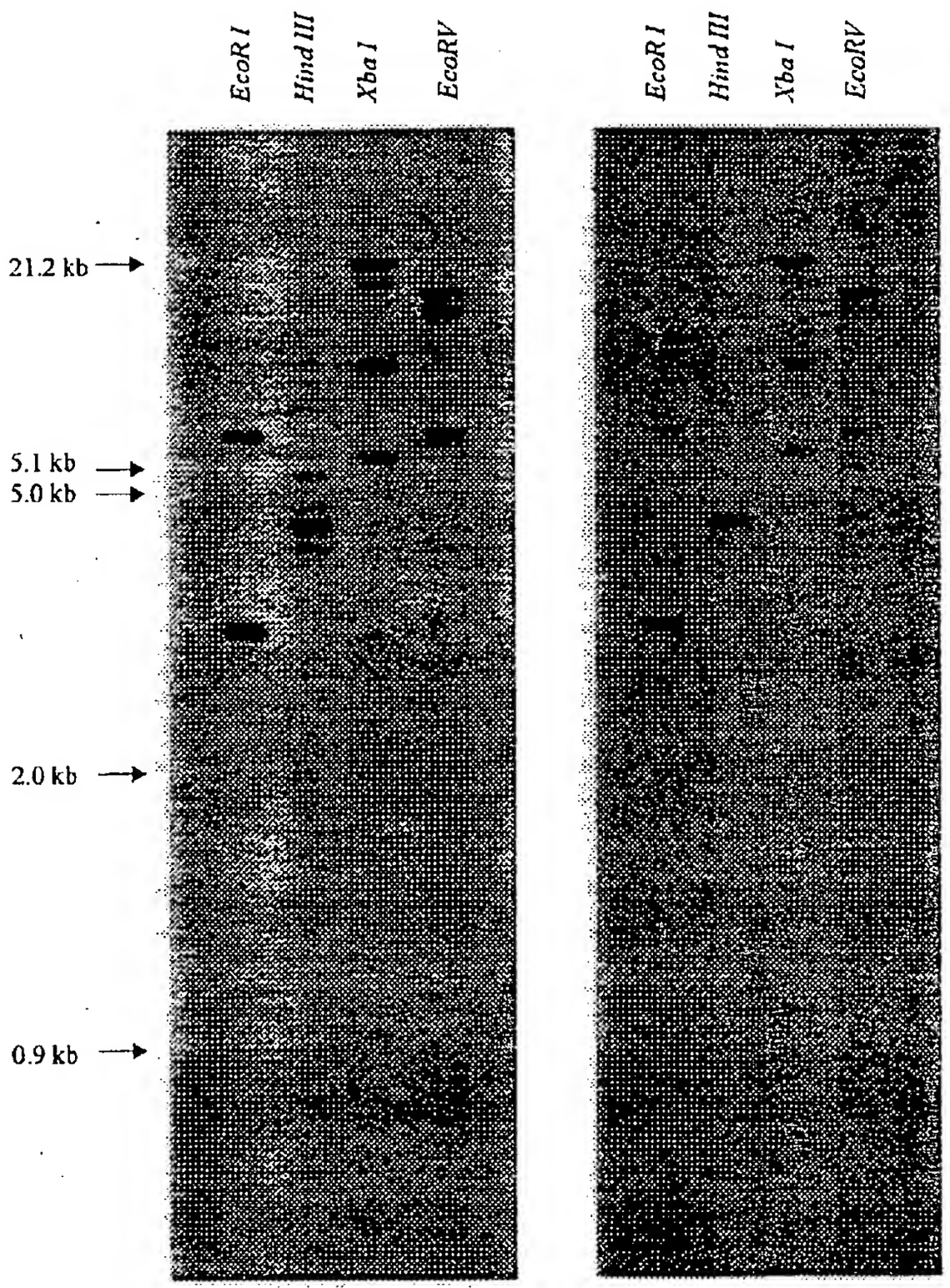

Fig. 25 A Fig. 25 B

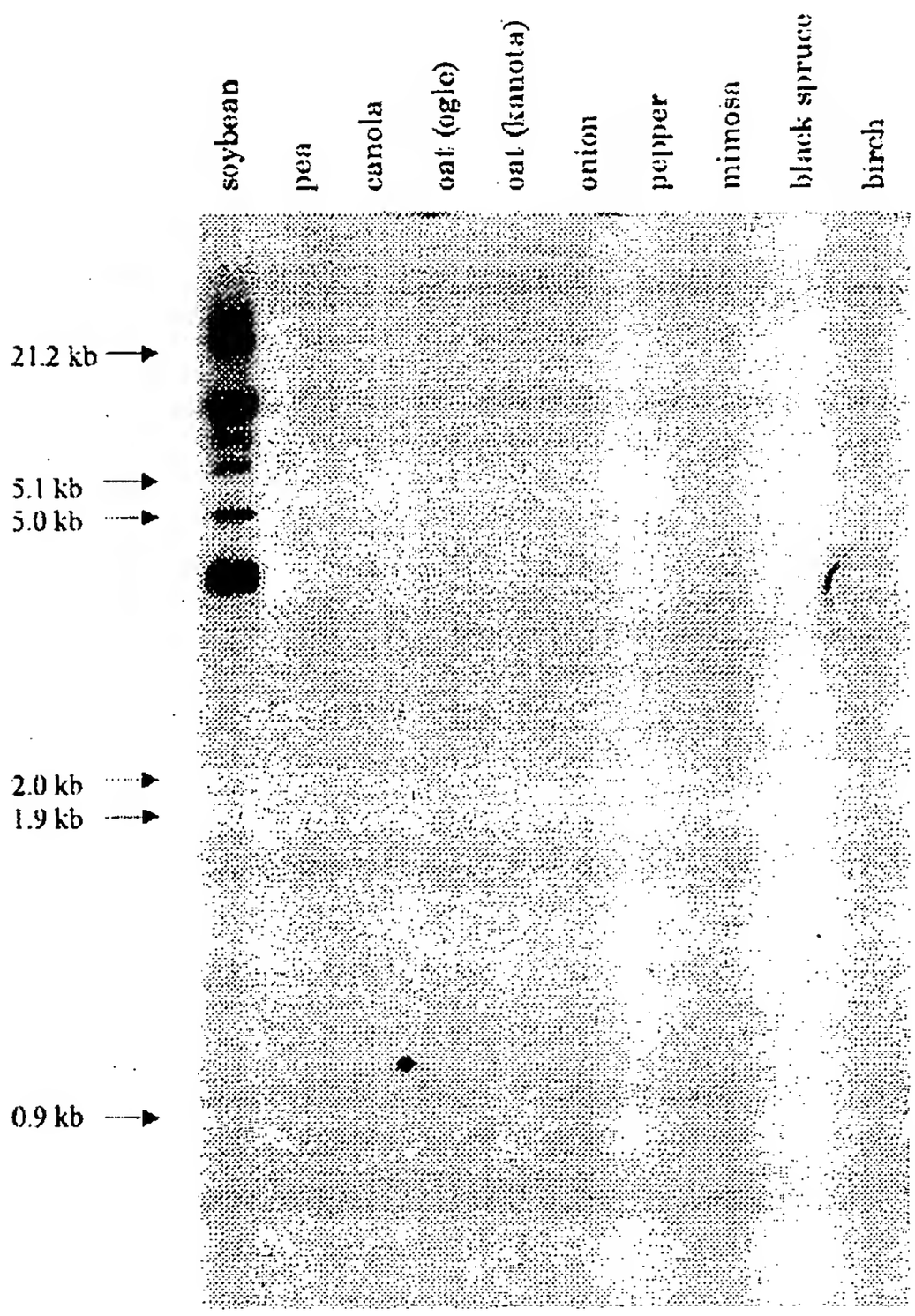
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Fig. 25 C

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Bowman, LuAnne
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WEST

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<213> Glycine max

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<211> 3368

WEST

<212> DNA

<213> Glycine max

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WEST

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